OSTEOACTIVIN AND CD44 : A NOVEL INTERACTION REGULATING BONE CELL DIFFERENTIATION AND FUNCTION

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LIST OF ABBREVIATIONS

ALP	Alkaline Phosphatase
BFR	Bone Formation Rate
Col1α1	Collagen Type I Alpha I
CTX-1	Carboxy-terminal Collagen Crosslink 1
CTX-B	Cholera Toxin-B
HA	Hyaluronan
KO	Knockout
MAR	Mineral Apposition Rate
MSC	Mesenchymal Stem Cell
OA	Osteoactivin
OCN	Osteocalcin
OPG	Osteoprotegrin
OSX	Osterix
RANKL	RANK ligand
rOA	Recombinant Osteoactivin
RUNX2	Runt-related Transcription Factor
WT	Wildtype

DEDICATION

I would like to dedicate this manuscript to all of the people that have always been there to help me when I needed them.

To my parents, Mark and Lisa for always being there when I needed it the most. For all the guidance and wisdom you have shared with me throughout the years. For all of the times you gave me a shoulder to cry on. For giving me your ears to listen to my rants and complaints. I am the person that I am today because of you.

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ABSTRACT

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OSTEOACTIVIN AND CD44 :
A NOVEL INTERACTION STIMULATING BONE FORMATION AND INHIBITING BONE RESORPTION

Dissertation Advisor: Fayez F. Safadi, Ph.D.

Osteoporosis is a growing problem for the elderly population. The bisphosphonate anti-resorptive class of drugs is the most commonly prescribed medication, however, the need for anabolic agents to stimulate bone formation is crucial. Osteoactivin was first discovered in bone using the *op* osteopetrotic mutant rat model. Our lab was the first to show that Osteoactivin stimulates bone formation in osteoblasts. Although several pieces of literature exist describing the role of Osteoactivin in both osteoblasts and osteoclasts, little is known about the signaling pathway involved in its function.

In this study, we used the recombinant Osteoactivin in culture to determine its effects on osteoblast and osteoclast differentiation *in vitro*. First, we added recombinant Osteoactivin to osteoblast and mesenchymal stem cells in culture and found an enhancement in osteoblast differentiation and function by ALP staining, activity, mineralization and upregulation of gene expression. We found that Osteoactivin binds to

and interacts with CD44 to stimulate osteoblast differentiation. Furthermore, we determined that CD44 deficient mice have a skeletal phenotype *in vivo* and *ex vivo* that results in less bone mass. Lastly, we found that Osteoactivin mediated enhancement of osteoblast differentiation is mediated through the CD44-ERK signaling pathway.

In our next study, we wanted to determine if the lipid raft is important for Osteoactivin mediated signaling in osteoblast. Our results have shown that Osteoactivin co-localizes within caveolin rich lipid rafts. Furthermore, Osteoactivin stimulates clustering of CD44 with Caveolin-1 and disruption of lipid rafts prevents this process. Lastly, we show that disruption of lipid rafts results in an inhibition of Osteoactivin mediated ERK signaling in osteoblasts.

In our last study, as opposed to the above role of Osteoactivin in osteoblast formation we wanted to determine the role of recombinant Osteoactivin during osteoclast differentiation and function. Our results reveal that recombinant Osteoactivin inhibits osteoclast differentiation and size in a dose dependent manner. Futhermore, we show that Osteoactivin reduces ERK and AKT phosphorylation in osteoclasts. The Osteoactivin mediated inhibition of osteoclasts was found to involve its interaction with CD44. Lastly, we show that recombinant Osteoactivin inhibits osteoclast migration and recruitment in wild type samples *in vivo*, and that this effect is abrogated in CD44 deficient mice. Overall, we believe that Osteoactivin and its interaction with CD44 results in the enhancement of osteoblast differentiation and an inhibition of osteoclast resorption.

Chapter 1

Introduction

The purpose of this proposed study was to observe the effects of recombinant Osteoactivin in both osteoblasts and osteoclasts using both *in vitro* and *in vivo* techniques. Furthermore, we wanted to determine a novel signaling pathway responsible for Osteoactivin-mediated function. Lastly, we wished to determine the effects of recombinant Osteoactivin *in vivo* using a calvaria osteolysis model. Our overall hypothesis is that Osteoactivin stimulates bone formation and inhibits bone resorption through the CD44 receptor. We believe that our results indicate that Osteoactivin may potentially be used as a therapeutic to treat bone loss and accelerate fracture healing.

Bone is a dynamic tissue consisting of a variety of cell types. The osteoblasts are the cells that secrete organic matrix and minerals that form bone. Osteoclasts are multinucleated cells that secrete degradative enzymes that result in the resorption of bone matrix. Through the use of genetically manipulated animal models the discovery of new molecules important for the treatment of bone diseases has exceedingly increased. Osteoactivin (OA) is a type I transmembrane protein that was first discovered in a model of the *op* osteopetrotic rat [1]. Since its discovery in bone there have been several publications describing the role of Osteoactivin in osteoblasts and osteoclasts.

Our lab has shown that Osteoactivin is a positive regulator of bone formation *in vitro* and *in vivo* [2-5].

Previously, we have shown that introduction of a nonsense mutation in the Osteoactivin gene resulting in a biologically inactive truncated protein of 150 amino acids at the N-terminal domain resulted in impaired bone formation both *in vivo* and *ex vivo* [6]. The mutation in Osteoactivin was shown to affect both cell types [6, 7]. For osteoblasts the mutation in Osteoactivin resulted in an inhibition of osteoblast differentiation and function [6]. For osteoclasts, mutation in Osteoactivin resulted in enhanced osteoclast differentiation but defective osteoclast resorption [7]. Therefore, we came to the conclusion that Osteoactivin acts as a positive regulator of osteoblast differentiation and function, a negative regulator of osteoclast differentiation, and a positive regulator of osteoclast function. Although our studies indicate that Osteoactivin is a negative regulator of osteoclast differentiation but a positive regulator of osteoclast function, other groups have shown that Osteoactivin acts as a positive regulator for both osteoclast differentiation and function [8-10]. The role of Osteoactivin should be clearly defined in order for its potential use as a therapeutic option.

As the aging population continues to increase, the prevalence of bone related pathologies will also continue to increase. According to the US Census Bureau, the number of people age 65 and older is expected to increase from 35 to 86 million, and people age 85 and older is expected to increase from 4 to 20 million between 2000 and 2050 [11]. Fractures seem to be the largest problem as they are common, costly, and have a substantial impact on the quality of life for the elderly population. There is an estimated 1.5 million individuals that suffer fractures caused by bone disease each year

[12]. It is expected that women over the age of 50 have a 40% risk of fracture which continues to increase with age [13]. Osteoporosis is the most common cause of fractures with an estimated 33.6 million individuals over the age of 50 having osteopenia or low bone mass [11]. There are other bone diseases that are much less common but still have a detrimental impact on the U.S. population.

An estimated one million individuals are diagnosed with Paget's disease, characterized by abnormal bone remodeling resulting in few or several sclerotic bone lesions within the body [14]. Furthermore, roughly 20-30 thousand individuals have Osteogenesis Imperfecta (OI), also known as brittle bone disease due to a mutation in the synthesis of type I collagen [13]. Patients with OI are at a higher risk for fracture due to decrease bone mass but also suffer several skeletal abnormalities including shortening of the limbs and severe kyphoscoliosis [15]. Osteopetrosis is a rare bone disease caused by a defect in bone resorption by the osteoclasts [16]. The resulting symptoms are abnormally dense bone, smaller medullary cavities affecting hematopoiesis, and nerve dysfunction. Experimental evidence gathered from animal models mimicking bone disease have been beneficial in the discovery of new therapeutics to help treat a variety of bone diseases. The search for new therapeutics is necessary to enhance the quality of life for those suffering from bone related pathologies.

Although the role of Osteoactivin in both osteoblasts and osteoclasts is becoming more defined, the signaling pathway involved in Osteoactivin-mediated stimulation of osteoblast differentiation and inhibition of osteoclast differentiation has yet to be determined. Previous literature has shown that Osteoactivin extracellular domain (ECD)

can be shed from the membrane by ADAM10 resulting in the release of a 500 amino acid biologically active molecule [17]. In this study, we used the recombinant Osteoactivin (which mimics the extracellular domain) to determine its effect on osteoblast and osteoclast differentiation and signaling. This study consisted of three specific aims and that tested our overall hypotheses that recombinant Osteoactivin is a positive regulator of bone formation by stimulating osteoblast and inhibiting osteoclast differentiation, respectively. We investigated this hypothesis using three specific aims.

Aim 1: To determine the role of recombinant Osteoactivin on osteoblast differentiation *in vitro* and identify a novel signaling pathway involved in Osteoactivin-mediated osteoblast differentiation and function.

Aim 2: To determine the role of recombinant Osteoactivin in the osteoblast lipid raft.

Aim 3: To determine the role of recombinant Osteoactivin on osteoclast differentiation *in vivo* and *in vitro* and identify a novel signaling pathway involved in Osteoactivin-mediated osteoclast differentiation and resorption.

This study was able to more clearly define the role of recombinant Osteoactivin in both osteoblasts and osteoclasts, and define a signaling pathway relating to its function. This study will be an important indicator for the use of Osteoactivin as a viable treatment option for osteoporosis and the acceleration of bone growth in cases of non-union fractures and spinal fusions.

Methodology

In this study several techniques were used to study the role of Osteoactivin *in vitro* and *in vivo*.

In Aim 1, the use of *in vitro* culture to assess osteoblast differentiation and function was determined. The two cell types used to assess osteoblast differentiation were the MC3T3-E1 osteoblast like cell line and bone marrow derived mesenchymal stem cells (MSCs) from mice. Other molecular techniques such as qPCR, Western blot, luciferase assay, immunofluorescence, and immunoprecipitation were used to determine the role of Osteoactivin in osteoblast signaling. Furthermore, several in vivo techniques were used to characterize the CD44 deficient (CD44KO) animals. These techniques include micro computed tomography (microCT; µCT), histology and histomorphometry, calcein labeling, and biochemical analysis of bone formation and resorption markers through the use of ELISA. Furthermore ex-vivo analysis using wild type (WT) and CD44 deficient cells was determined in order to observe the relationship between CD44 and Osteoactivin.

In Aim 2, the use of *in vitro* techniques such as immunofluorescence and Western blot were used in order to determine the role of Osteoactivin with the lipid raft. Futhermore, the use of sucrose-gradient ultracentrifugation was used in order to isolate lipid raft fractions.

In Aim 3, the use of *in vitro* techniques such as TRAP staining and activity, qPCR, Western blot, immunofluorescence, and ELISA were used to determine the role of Osteoactivin in osteoclast differentiation and signaling. Furthermore, we used an *in*

vivo osteolysis model to determine the role of Osteoactivin in osteoclast differentiation and recruitment.

Limitations

In this study, we used the recombinant extracellular domain of the Osteoactivin protein. Therefore, the effects that we observed are strictly due to the extracellular domain, and may not mimic the same effects of the entire molecule. Furthermore, the osteolysis model used in Aim 3 only mimics what is occurring within the local environment. Systemic injections of either RANKL or Osteoactivin were not given due to cost, dosing and timing therefore, we cannot conclude that our model would show similar results if used systemically.

Chapter 2

Literature Review

2.1 The Skeleton

The mature adult human skeleton consists of 213 bones: 126 from the appendicular skeleton, 74 from the axial skeleton, and 6 auditory ossicle bones [18]. Throughout the course of life, bone undergoes constant remodeling which can be influenced by a variety of factors, including genetics, aging, sex, biomechanical forces, and dietary habits (Figure 2.1). The skeleton serves a variety of unique functions. These functions include movement and structural support, protection of vital organs, regulation of mineral homeostasis, a reservoir for certain growth factors and cytokines, and provides an environment for hematopoiesis within the bone marrow microenvironment [19]. There are four general categories of bone. These include long bones (i.e. femur, tibia, humerus, etc.), short bones (carpals, tarsals, patellae, and sesamoid bones), flat bones (craniofacial bones, sternum, and ribs), and irregular bones (vertebrae, coccyx, sacrum, and hyoid bones) [20].

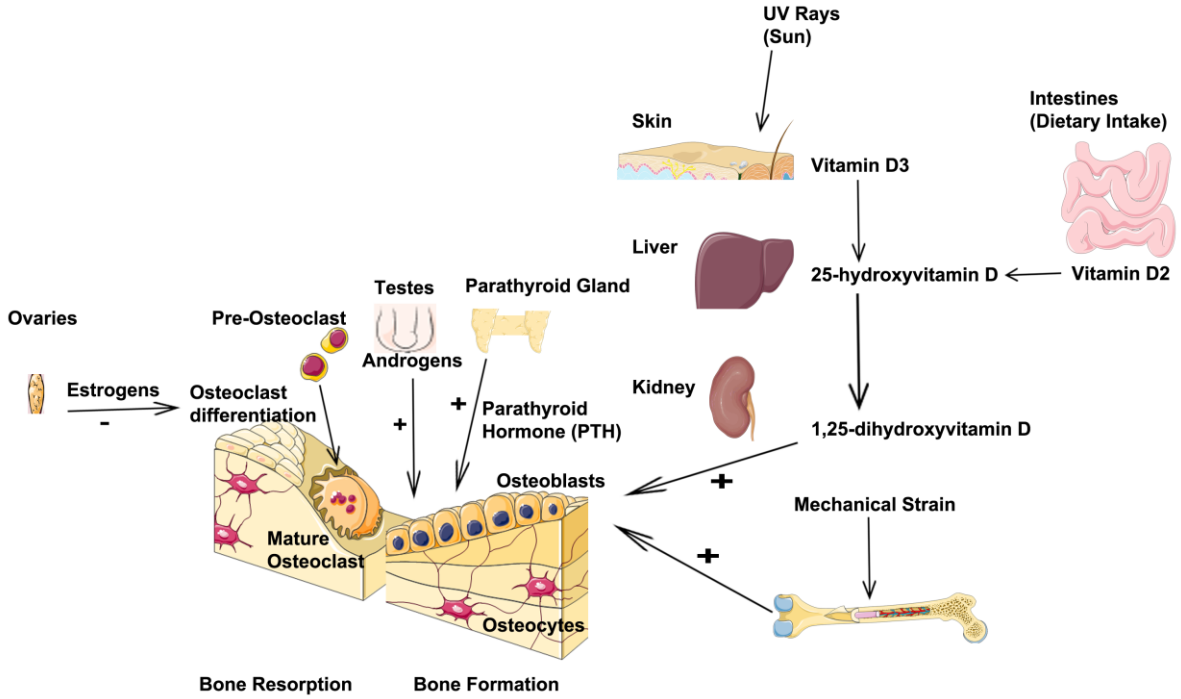


Figure 2.1: Schematic diagram indicating the effects of hormones, dietary intake, and mechanical strain on the regulation of bone mass. Several factors influence bone homeostasis and remodeling. The above diagram indicates the role of several important hormones and vitamins including estrogens, androgens, PTH and vitamin D and their effects on osteoblasts and osteoclasts. Furthermore, mechanical strain also plays a major role in the regulation of bone formation.

The external anatomy of long bones consists of three parts. The longest portion consists of a hollow medial diaphysis, which houses the bone marrow; the cone-shaped metaphysis below the growth plate; and the epiphyses, which sits directly above the growth plates. Bone is generally categorized into two different types: cortical or compact bone and trabecular bone, also known as cancellous or spongy bone. In general, the human skeleton is comprised of 80% cortical and 20% trabecular bone; however, the ratio can change based on the bone observed [20]. Cortical bone is much denser than trabecular and surrounds the marrow space, whereas trabecular bone has a sponge or honeycomb-like network composed of interspersed bone (Figure 2.2).

Trabecular bone is much more porous than cortical bone. Trabecular bone consists of roughly 50-95% porosity in which the spaces are primarily filled with marrow, and the remaining bone matrix has the form of plates referred to as the trabeculae [21]. Cortical bone porosity in general is roughly 5% and contains within it Haversian systems or Osteons. Haversian systems are formed of concentric lamellae along their walls and align with the long axis of the bone. Within the Haversian system are osteocytes which are housed within the lacunae and communicate with other cells by canaliculi [22]. Volkmann's canals are also located within the cortical bone and function as transverse canals connecting other Haversian canals (Figure 2.4). Blood vessels and nerves are located in both the Haversian and Volkmann's canals.

Cortical bone contains an outer periosteal layer and an inner bone marrow facing endosteal surface. The periosteum contains a fibrous connective tissue layer with an inner cambium layer in direct contact with the cortical bone [23]. The inner cambium layer is highly cellular, which contains osteoblasts, fibroblasts, mesenchymal progenitor

cells, as well as blood vessels and nerves [24]. The outer fibrous layer consists primarily of collagen and elastin fibers important for other connective tissue and muscle attachment. Both layers have been shown to be important for fracture healing and appositional growth. The endosteal surface, or endosteum, covers the inner surface of the cortical bone in direct contact with the marrow space. It consists of osteoblasts, osteoclasts, bone lining cells, nerves, and blood vessels.

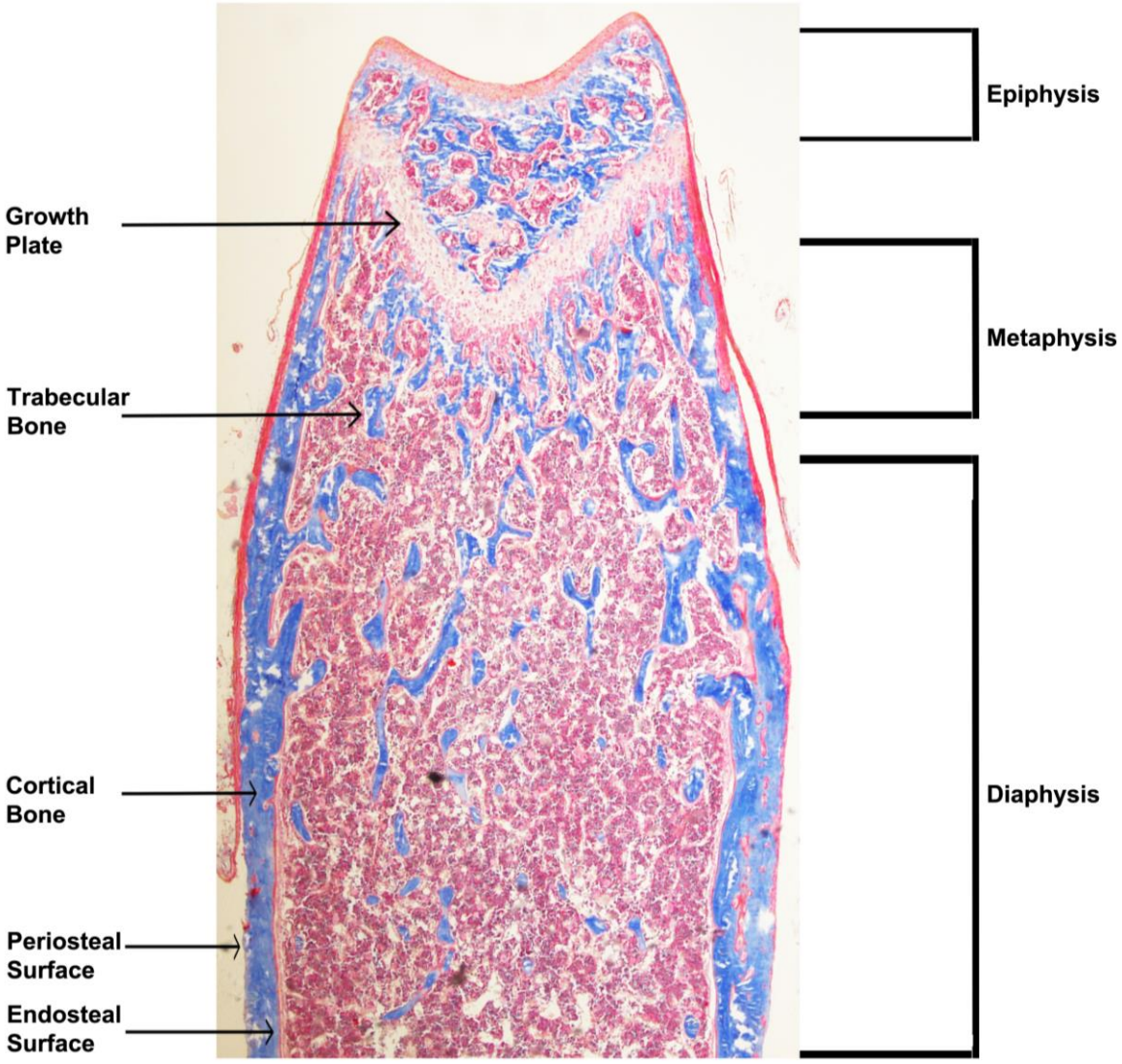


Figure 2.2: Bone histomorphology of the distal femur using Masson's trichrome staining. Histological image depicting the distal femur of a C57Blk6 8 week old mouse. The collagen (blue) indicates areas of both trabecular and cortical bone. Furthermore, both outer periosteal and inner endosteal surfaces of cortical bone are shown along with the three primary regions of long bones (epiphysis, metaphysis, and diaphysis). Sections of the distal femur were imaged at 2X magnification.

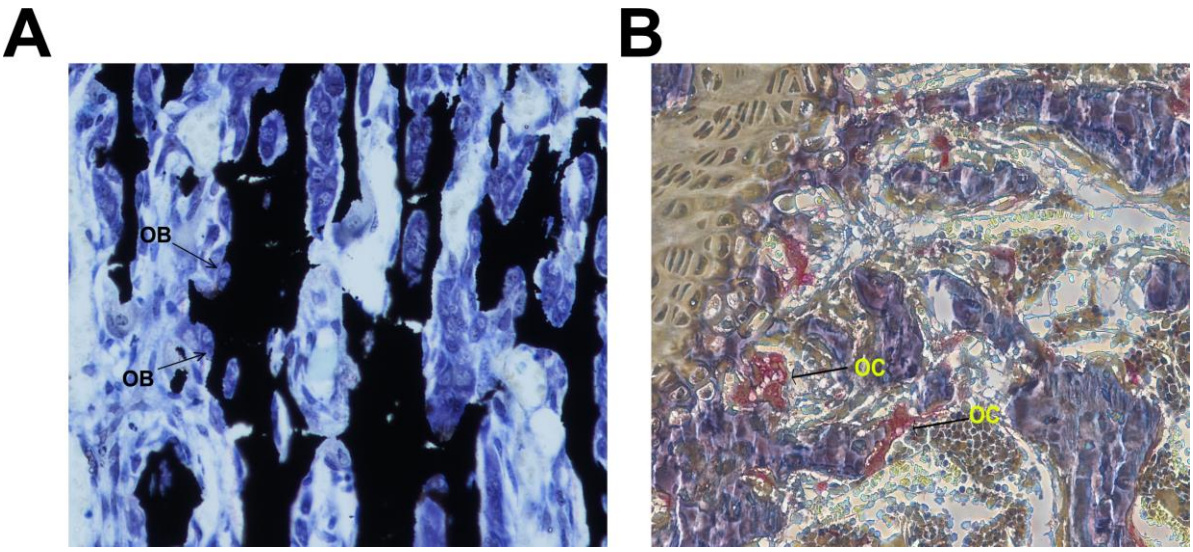


Figure 2.3: Bone histomorphology of the osteoblast and osteoclast. (A) Von kossa staining of the distal femur from an 8 week old C57Blk6 male mouse. Notice the osteoblasts (OB, arrows) sit on the trabecular bone surface (black) where they deposit extracellular bone matrix and minerals. **(B)** Tartrate resistant acid phosphatase (TRAP) staining of the distal femur from an 8 week old C57Blk6 male mouse. Notice the osteoclasts (OC, red, arrow) also sit on the trabecular surface (green) where they secrete degradative enzymes that break down bone matrix. Sections of the distal femur **(A and B)** were imaged at 20X magnification.

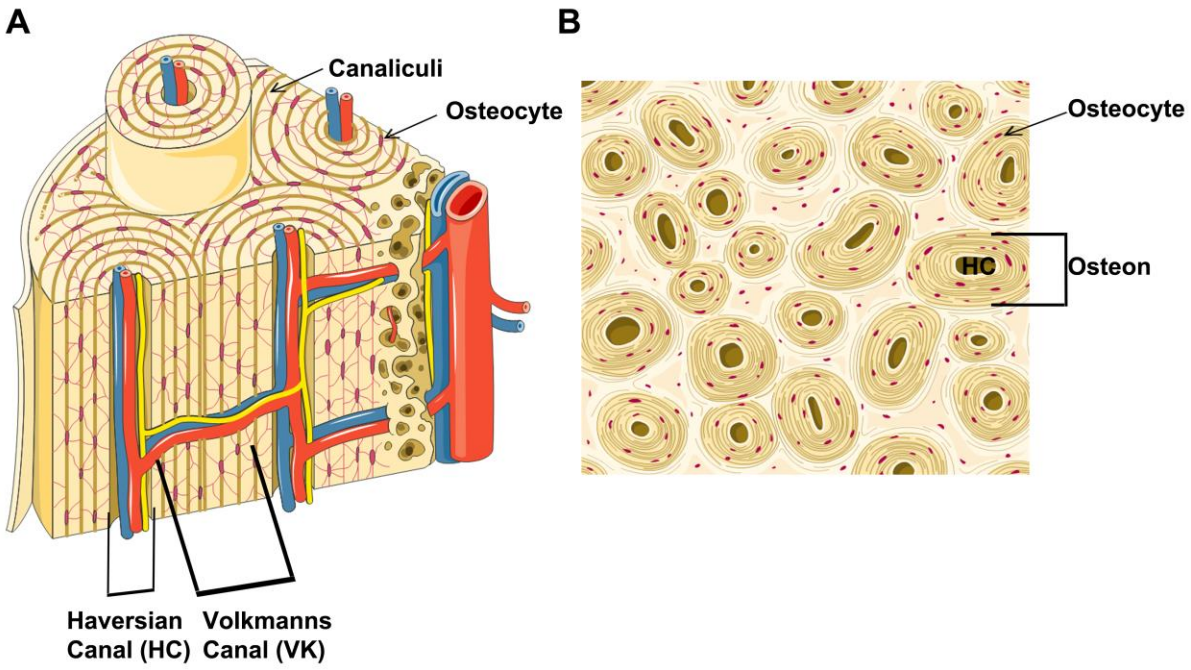


Figure 2.4: Schematic diagram of the osteon, Haversian canals, and Volksmann canals in cortical bone. (A) Longitudinal section of cortical bone depicting the Haversian and Volkmanns canals along with osteocytes (below arrow, pink cells) and connecting canaliculi (above arrow, pink lines). Notice the Haversian canals run in parallel with the cortical bone whereas the Volkmanns canals run transverse. **(B)** Cross-section of cortical bone depicting the osteons that contain the osteocytes housed within lacunae and communicate by canaliculi. Notice the Haversian canal in the center of the osteon.

2.2 The Composition of Bone

Bone is a composite material consisting of a variety of molecules that include minerals, organic matrix, water, and lipids [25]. The bone structure relies on several factors including sex, age, dietary status, and health. The structural differences seen in skeletal sexual dimorphism can be attributed to the opposing sex steroid actions that differ among men and women [26]. The changes in skeletal structure associated with aging can be attributed to the loss of the homeostatic mechanisms that regulate bone remodeling. These include changes in hormones, biochemical factors, and genetics [27]. Dietary intake of several vitamins and minerals such as vitamin D and calcium are critical for proper skeletal development [28]. Lastly, health status can have a crucial impact on skeletal maintenance. For example both diabetes [29] and cancer [30] can have negative impacts on bone homeostasis. All of these factors influence the structure and composite of bone. This aspect is important as proper maintenance of bone structure and composition allow it to carry out its several mechanical and metabolic functions.

The minerals of bone are a complex system composed primarily of calcium, phosphate, magnesium, and carbonate [31]. The most highly, naturally occurring mineral within the bone microenvironment is an analog of hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] [32]. The mineral phase of bone has several functions. These functions include providing mechanical resistance to the tissue by strengthening the collagen matrix and serving as a reservoir for mineral homeostasis for ions including calcium, phosphate, and magnesium [33]. Bone hydroxyapatite, also referred to as bone apatite, contains crystals that can be found in a variety of sizes. During the

remodeling phase it is the smaller crystals that undergo physiochemical reactions that result in the release of ions due to the resorptive activity of the osteoclasts [32]. The size and nature of the apatite crystals can serve as a diagnostic indicator for bone diseases such as osteoporosis and osteopetrosis. In the case of osteoporosis, larger apatite crystals are present within the bone matrix in greater abundance, which contribute to the brittle bone phenotype [34]. In the case of osteopetrosis in which bone remodeling is impaired, the apatite crystals are relatively small compared to age-matched controls.

Type I collagen serves as the basic building block of the bone matrix network and consists of roughly 85-90% of the total bone protein content [35]. Type I collagen consists of three polypeptide chains that form a triple-helix structure [36]. The helix structure contains two identical α1 (I) chains and a structurally similar α2 chain [37]. Collagen provides strength and elasticity for the bone, and its structure directs organization of the bone matrix. The collagen fibers are oriented in one of two ways: in the case of periosteal bone, collagen fibers are organized in sheets, and in osteonal bone, they are organized circumferentially [25]. In the collagen-mineral interface, the long axes of apatite crystals are aligned parallel to the collagen fiber axis (Figure 2.5) [38]. This orientation allows the minerals to contribute strength to the collagen matrix. Collagen undergoes several post-translational modifications that determine its structural and mechanical components within the bone microenvironment. Two examples of these post-translational modifications include hydroxylation of lysine residues and glycosylation of hydroxylysine [39-41]. The proper modifications to collagen are important for its biomechanical properties such as toughness and ductility.

Abnormalities in the cross-linking of collagen fibers have been used as a diagnostic indicator for several bone diseases including Osteoporosis, Osteogenesis Imperfecta, and Ehler's-Danlos syndrome [35]. Collagen is rapidly and frequently degraded within the bone extracellular matrix. Cleaved products of collagen can also act as diagnostic indicators in such cases of rapid bone resorption or formation. In the case of bone formation, Procollagen type 1 amino terminal propetide (P1NP), cleaved by procollagen peptidases, serves as specific byproducts of osteoblasts during collagen formation [42]. In the case of bone resorption, Carboxy-terminal cross-linked telopeptide of type I collagen (CTX-1), cleaved by enzymes such as Cathepsin K and matrix metalloproteinases (MMPs), serves as byproducts of osteoclast activity during bone resorption [43, 44]. The structure of collagen also allows for the interaction with non-collagenous matrix proteins. Collagen along with other non-collagenous proteins have shown to serve to regulate the size and orientation of mineral deposits within the bone microenvironment [45].

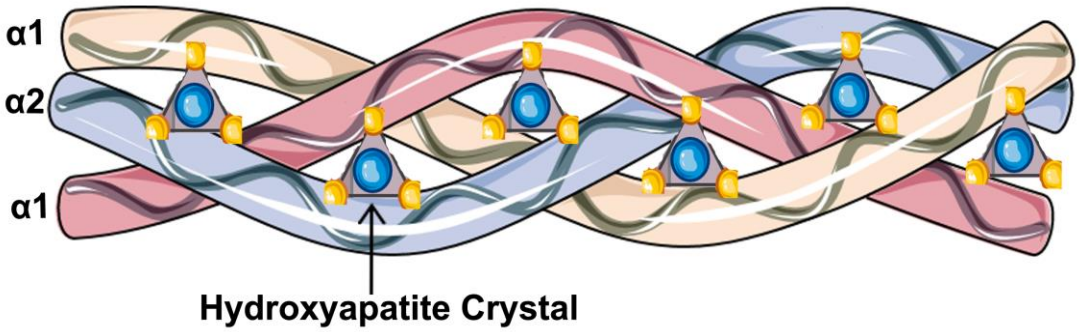


Figure 2.5: Schematic diagram depicting the orientation of collagen fibers and hydroxyapatite minerals in bone. Type I collagen found in bone consists of three polypeptide chains, two of which are identical, that form a triple helix. In the collagen-mineral interface, hydroxyapatite crystals are oriented parallel along the collagen fibers. The orientation of the collagen-mineral interface is crucial for maintaining the proper biomechanical properties of bone.

Noncollagenous proteins (NCPs) consist of a wide variety of proteins that comprise roughly 10-15% of the bone matrix. These proteins have a wide variety of functions, and their discovery has primarily arisen from the use of knockout or transgenic animals. The NCPs of bone matrix are categorized primarily based on their structural properties and their function to bone.

Serum-derived proteins comprise about 25% of the NCPs found in bone matrix and, due to their acidic nature, have high affinity for binding to hydroxyapatite [32]. These proteins are not endogenously synthesized within the cell; however, they have shown to play a role in both matrix mineralization and cell proliferation. An example of a serum derived protein that plays a role in bone is α_2-HS-glycoprotein. The knockout animal of this serum derived protein exhibits ectopic calcification, indicating α_2-HS-glycoprotein plays a role in the regulation of matrix mineralization [46]. There are a variety of other NCPs present in smaller trace amounts that have shown to play a role in matrix development. These NCPs include proteoglycans, glycosylated proteins, and γ-carboxylated (gla) proteins.

Proteoglycans are large macromolecules with a central core protein and acidic polysaccharide side chains (glycosaminoglycans) [47]. There are several bone proteoglycans all with a variety of functions. During bone formation chondroitin sulfate and hyaluronan are highly expressed proteoglycans that make up the foundation for areas that will eventually become bone [48]. Several proteoglycans contain a leucine-rich repeat sequence that relates to their specific function in bone. Two examples of these proteoglycans are Decorin and Biglycan. Decorin has been shown to play a role in collagen regulation, whereas Biglycan has been shown to be a positive regulator of

bone formation as the knockout animal has shown to have decreased trabecular bone [49]. In addition there are also smaller proteoglycans that have a rich leucine repeat (SLRP) that play a role in bone. These SLRPs include Osteoadherin, Osteoglycin, and Fibromodulin. The functions of these proteins in bone are still relatively unknown; however, it is thought they may play a role in maintaining the integrity of connective tissue matrices and modulate the activity of certain growth factors and cytokines in the extracellular space [50, 51].

Glycoproteins play a very diverse, multifunctional role within the bone matrix. Alkaline Phosphatase (ALP) is an important bone glycoprotein that has been used as a marker to assess bone formation. Lack of ALP has been linked to the human disease hypophosphatasia, in which patients have significant decreases in mineralized bone (hypomineralization) [52]. Although the specific role of ALP has not been clearly defined, it is believed that ALP hydrolyzes mineral inhibitor pyrophosphates (PPi) which yields monophosphate ions important for bone mineralization [53]. Therefore, in the case of ALP deficiency, inhibitory pyrophosphates accumulate into matrix vesicles resulting in an inhibition of mineralization of bone. Osteonectin is also a very abundant glycoprotein found within the bone matrix. It is the most abundant non-collagenous protein in the bone matrix (~2%), and mice deficient in Osteonectin are severely osteopenic [54]. Osteonectin has been shown to play a role in the regulation of both osteoclasts and osteoblasts. A previous report has shown that osteonectin seems to play a large role in osteoblast proliferation and matrix mineralization [55].

Several glycoproteins within the matrix have several unique domains, which include sulfation sites, phosphorylation sites, large sugar moieties, and RGD domains.

These glycosylation sites have several roles within the matrix; however, one of the more important roles of these domains involves cell attachment through focal adhesions to other extracellular macromolecules within the matrix through integrin binding [56]. Many of these glycosylation sites occur on the N-terminal side of the protein and belong to a family of proteins known as the SIBLING (small integrin-binding ligand, N-glycosylated proteins) family which include Bone Sialoprotein, Osteopontin, and Dentin Matrix Protein [32]. Bone Sialoprotein (BSP) is a 34 kDa protein with an RGD domain that plays a role in matrix mineralization [57, 58]. In 16 week BSP knockout mice, it was found that the animals had higher trabecular bone density and lower bone turnover with shorter femurs, smaller body length, and thinner cortices [59]. BSP knockout mice also had dysfunctional osteoblast and osteoclast differentiation which indicates that BSP plays a role in both cell types [60, 61]. Osteopontin (OPN, SPP1) is a phosphorylated SIBLING family member that shows similar structure and function as BSP. Previous work has shown that both OPN and BSP bind to the $\alpha_v\beta_3$ integrin in osteoclasts and are important for osteoclast bone resorption *in vitro* [62]. OPN knockout mice have been shown to develop normally [63]; however, ovariectomized mice that resemble post-menopausal osteoporosis are resistant to bone loss indicating OPN plays a major role in bone in post-menopausal women [64]. Dentin Matrix Protein-1 (DMP-1) is a cleaved SIBLING protein expressed predominantly in osteocytes and odontoblasts of the teeth [65]. DMP-1 knockout mice have shown to display the human condition autosomal recessive hypophosphatemic rickets with severe osteomalacia and hypocalcemia [66]. This indicates DMP-1 plays a large role in the mineralization process. DMP-1 has been

found to regulate skeletal mineralization through its regulation of fibroblast growth factor-23 (FGF-23).

There are several other RGD proteins that play a primary role in cell attachment. Fibronectin is a highly expressed protein in bone that has shown to have a dual role in the differentiation of both osteoblasts and osteoclasts. Fibronectin as an extracellular matrix protein has shown to play a positive role in osteoblasts [67, 68] by regulating mineralized nodule formation through integrin receptors $\alpha_5\beta_1$ and $\alpha_8\beta_1$. Fibronectin also plays a role in osteoclasts by inhibiting their differentiation, but stimulating their function through nitric oxide and IL-1β signaling [69]. A similar RGD adhesion protein that plays a role in bone is Vitronectin. Vitronectin has shown to stimulate osteogenic differentiation through integrin receptors [70]. Vitronectin has shown to play a major role in osteoclast resorption through its primary receptor $\alpha_v\beta_3$. Furthermore, Vitronectin has shown to play a major role in podosome and ruffled border formation and in the release of tartrate-resistant acid phosphatase (TRAP) in osteoclasts [71]. Fibrillin-1/2 are extracellular matrix proteins that interact with and regulate other growth factors and cytokines in the bone matrix. Fibrillin-1 mutations have been linked to the human disease Marfan syndrome in which patients suffer from cardiovascular, eye, lung, and skeletal defects [72]. Marfan syndrome patients have decreased bone mineral density (BMD) in the femur and spine. Fibrillin-1 mice are neonatally lethal [73], and Fibrillin-2 knockout mice have significantly less bone compared to wild types due to their ability to modulate bioavailability of transforming growth factor-β (TGF-β) and bone morphogenetic proteins (BMPs).

A small subset of NCPs in bone matrix that are regulated by Vitamin-K dependent carboxylases are members of the Gamma-Carboxy Glutamic Acid or Gla proteins. The Gla residues are important for hydroxyapatite binding and critical for these subsets of proteins to function. Two specific Gla proteins that have been extensively studied are Matrix Gla Protein (MGP) and Osteocalcin (bone Gla protein; BGP;OCN). MGP is expressed primarily in vascular smooth muscle cells and chrondrocytes [74] and has been shown to be an inhibitor of extracellular matrix mineralization. Mutations in the MGP gene have been shown to be linked to the human disease Keutel syndrome, a rare autosomal recessive condition in which patients show symptoms of abnormal calcification in soft tissues [75]. MGP deficient mice have a short life span of 8 weeks due to excess vascular calcification leading to vessel rupture [76]. MGP mice also have premature mineralized growth plates and have significantly less bone due to impaired osteoblast function [74, 77]. Osteocalcin is expressed in both mature mineralizing osteoblasts and osteocytes [78, 79]. Osteocalcin is a circulating molecule thought to be a marker for bone formation and has been used as a clinical maker for testing bone mineral density [80, 81]. Osteocalcin deficient mice develop normally until 6 months of age and thereafter begin to develop in osteopetrotic phenotype [82]. Although the exact mechanism of Osteocalcin is still unclear, it is believed to be a signal in the bone turnover cascade between bone formation and bone resorption.

There are several other elements within the bone microenvironment that play a role in bone homeostasis. Many enzymes and growth factors are located within the bone matrix or on the cell surface and play a major role in bone homeostasis. Water accounts for roughly 10% of the bone volume and serves a variety of functions,

including control of ion flux, cell and matrix nutrition, and maintenance of collagen structure [32]. Water has been used to test the mechanical properties of bone. It has been well documented that following the dehydration of bone, the stiffness, tensile strength, and hardness increase while the strain and energy to fracture decreases [83]. Lipids also play a role in bone homeostasis. Several factors that influence lipid metabolism such as Sphingomyelinase [84, 85], Caveolins [86], and Phospholipase D [87] all have unique effects on bone development.

Sphingolipid biosynthesis and metabolism has been shown to be an important regulator of skeletal development [85, 88]. In a previous study, deletion of the gene encoding sphingomyelin phosphodiesterase 3 resulted in Osteogenesis Imperfecta indicating the importance of sphingomyelinase enzymes in bone homeostasis [85]. Sphingomyelinase enzymes are responsible for the cleavage of sphingomyelin which generates ceramide and phosphocholine [89]. Ceramide itself has been shown to induce apoptosis in osteoblasts; however, ceramide derivatives sphingosine and sphingosine-1-phosphate (S1P) have been shown to enhance osteoblast migration and early stage osteoblast differentiation [90, 91].

Recent evidence suggests that dysfunction of enzymes that influence lipid metabolism may also influence bone metabolism. Previous literature has shown that an association between lipid and bone metabolism disorders such as atherosclerosis and osteoporosis exist [92, 93]. Furthermore, increase in adipocyte levels correlate with a decreased level of bone mineral density and increased bone loss. During aging, the hyperaccumulation of lipids leads to the pathogenesis of athlerosclerosis and osteoporosis. One of the primary class of drugs used to treat athlerosclerosis are

members of the statin family (Simvastatin, lovastatin) which act to lower cholesterol by inhibiting the enzyme 3-hydroxy-3-methyl-glutaryl-CoA reductase important for the cholesterol biosynthesis pathway [93]. Interestingly statins have been shown to have a positive effect on bone formation and bone mineral density [94, 95]. Previous literature has shown that statins positively affect osteoblast differentiation and function and negatively affect osteoclast proliferation and differentiation [96-98].

2.3 Cellular and Molecular Determinants of Bone

Mesenchymal stem cells (MSCs; marrow stromal cells) are pluripotent cells located throughout the body. Pluripotent stem cells (PSC's) in the bone marrow have the potential to differentiate into multiple cell lineages, including muscle, bone, cartilage, adipose, ligaments, and tendons [99, 100]. The potential for these cells to differentiate towards a certain lineage is based upon the cytokines, growth factors, and transcription factors specific to that tissue. The ability for MSCs to differentiate into a variety of cell lineages makes them an ideal candidate to help regenerate a variety of connective tissues. Several potential applications for the use of stem cell therapies for regenerative medicine have already begun [101]. In several animal studies, MSCs expanded *ex vivo* and placed back into the animal were able to differentiate into cells of the residing tissue, repair the damaged tissue, and help restore function in tissues including bone and cartilage [102, 103]. The use of MSCs in orthopedic medicine is of great interest due to the growing rate of osteoporosis and osteoarthritis. MSCs hold a potential role to help accelerate bone healing in cases of non-union fractures. In order for MSCs to play a role in bone regeneration, they must first have the ability and potential to differentiate into the bone matrix and mineralizing cells, the osteoblasts.

Osteoblasts are bone matrix secreting cells arising from the mesenchymal stem cell lineage. In order for MSCs to differentiate into osteoblasts, certain environmental cues that include cytokines, growth factors, and transcription factors must be expressed [104]. Osteoblasts undergo specific stages of differentiation which include proliferation, matrix deposition, matrix maturation, and mineralization (Figure 2.6) [105]. During proliferation osteochondrogenic precursors and pre-osteoblasts will continue to mitotically divide until given the molecular cue to stop. Once proliferation has ceased, immature osteoblasts begin secreting organic bone matrix proteins comprised primarily of type I collagen in a process known as matrix deposition. The unmineralized bone matrix during this stage is known as osteoid. After the osteoid layer has been secreted, mature late-stage osteoblasts begin depositing mineralized matrix composed primarily of hydroxyapatite [106, 107]. After the mineralization process there are three routes osteoblasts can undergo: they can become embedded within the bone matrix and transition into osteocytes, become bone lining cells, or undero apoptosis [108]. There are several markers that are used to assess different stages of osteoblast differentiation which include, ALP, Osteocalcin, type I collagen, Bone Sialoprotein, Osteopontin, and PTH [109]. These markers are transcriptionally regulated by several factors; however, the most well studied transcription factors controlling different stages of osteoblast differentiation are Runx2 and Osterix.

Runx2 (Osf2/Cbfa1) is a master transcriptional regulator of osteoblast differentiation expressed only in bone, which was discovered from the promoter analysis of the osteocalcin gene 2 (OG2) [110]. Runx2 belongs to the Runx family of transcription factors homologous to Runt, a Drosophila gene product [111]. Mice

deficient in Runx2 fail to form hypertrophic chondrocytes and completely lack osteoblasts as the mice only produce a cartilaginous skeleton that is completely devoid of bone matrix [112]. Mutations within the Runx2 gene in humans lead to a rare disease known as Cleidocranial Dysplasia characterized by complete or partial absence of the collar bones and defective development of the cranial bones [113]. Since its critical role in bone was first discovered, Runx2 has been shown to interact with other transcriptional regulators and signaling molecules important for bone formation [114]. These interactions can either be stimulatory or repress osteoblast specific genes that include type I collagen, Osteocalcin, Osteopontin, ALP, Osteonectin, and Bone Sialoprotein.

Osterix (Osx; Sp7) is a zinc finger transcription factor, critical for osteoblast differentiation. In mice lacking Osterix there is a complete absence of bone indicating that Osterix acts as a master transcription for bone formation [115]. Similar to Runx2, Osterix has been shown to interact with and regulate several important factors important for osteoblast differentiation. Previous literature has shown that the Osterix promoter contains a Runx2 binding domain, indicating that Runx2 acts upstream and regulates Osterix expression in osteoblasts [116]. There are several cytokine and growth factors that stimulate signaling pathways that mediate both Osterix and Runx2 activity and drive osteoblast differentiation and function. These include members of the BMP, TGF-β, Wnt, and FGF families.

Bone morphogenetic proteins (BMPs) are members of the TGF-β superfamily that were first detected in extracts taken from bone at ectopic sites [117, 118]. BMPs are ubiquitously expressed in a variety of tissues, including bone, and have shown to

play a critical role in osteoblast differentiation. Abnormalities in the BMP signaling pathway in humans can lead to the disease Fibrodysplasia Ossificans Progressiva (FOP) in which mutation in the BMP receptor leads to active bone formation at ectopic sites [119]. BMP signaling is stimulated through the binding of its heterodimeric serine/threonine kinase receptor (BMP receptors I (BMPR-I) and BMP receptors II (BMPR-II) complex which results in downstream Smad signaling. Upon stimulation of BMP, constitutively active BMPR-II phosphorylates and stimulates BMPR-I, which subsequently stimulate regulatory Smad 1, 5, and 8 by phosphorylation at their C-termini [120]. The receptor stimulated Smads associate with Smad 4 which then translocates to the nucleus and interacts with other transcription factors and bind to promoters of osteoblast specific genes. Previous literature has shown that BMPs cooperate with Runx2 and regulate expression of genes related to osteoblast differentiation and function [121, 122]. Interestingly, BMP2 was shown to induce Osterix expression through the MAPK pathway and stimulate osteoblast differentiation and function, indicating that BMPs are capable of interacting with and stimulating multiple signaling pathways related to osteoblast differentiation [123, 124].

Transforming growth factor (TGF-β) family of cytokines are largely abundant within the bone matrix and have shown to have both a negative and positive effect on bone formation. The signaling pathway for TGF-β is similar to that of BMPs; however, upon stimulation of the TGF-β type I receptor, regulatory Smads2 and 3 are activated and interact with Smad 4 to regulate transcription of osteoblast related genes [125, 126]. Interestingly, activated Smad3 inhibits Runx2 activation and expression resulting in decreased Osteocalcin expression and osteoblast mineralization [127]. Previous

literature has shown that TGF-β can also stimulate recruitment and proliferation of osteoblasts [128, 129], indicating a dual role for TGF-β during osteoblast differentiation.

The Wnt family of proteins are secreted glycoproteins that regulate several cellular functions through activation of its 7 transmembrane receptor frizzled and co-receptor LRP5/6 [130]. There are several downstream mediators of Wnt signaling. In the absence of Wnt, the transcription factor β-catenin complexes with Adenomatous Polyposis Coli (APC), Glycogen Synthase Kinase-3 (GSK3), Casein Kinase-1 (CK1), and Axin phosphorylating β-catenin tagging it for degradation [131]. When Wnt signaling is active, β-catenin does not get phosphorylated and instead accumulates in the nucleus where it regulates transcription of certain target genes along with other co-transcription factors TCF/Lef1. Wnt signaling has been shown to be a critical pathway during osteoblast differentiation.

The importance of Wnt signaling in bone came from studies using lipoprotein receptor-related protein (LRP) knockout and transgenic mice. LRP5 and LRP6 are part of a family of low density lipoprotein co-receptors for the Wnt family of ligands [132]. LRP5 deficient mice at 8 weeks of age were shown to have a significant reduction in trabecular bone volume due to a reduction in mineral formation and osteoblast proliferation [133]. Further evidence of the importance of LRP5 has been linked to the human disorder Osteoporosis Pseudoglioma Syndrome (OPPG), in which patients with a mutation in LRP5 resemble an osteoporotic phenotype [134, 135]. Similarly LRP6 deficient mice also reveal an osteoporotic phenotype. LRP6 homozygous knockouts are lethal at birth containing severe developmental abnormalities in the skeleton; however, the LRP6 heterozygous mice are viable but still exhibit significant decreases in bone

mineral density at 9 weeks age [136, 137]. Furthermore, LRP5$^{-/-}$ and LRP6$^{+/-}$ double knockout mice have a more dramatic phenotype than LRP5$^{-/-}$ or LRP6$^{+/-}$ mice alone, indicating that LRP5 and LRP6 co-operate and interact with each other to regulate bone mass [138]. There have been several studies revealing the importance of the β-catenin transcription factor during osteoblastogenesis. Conditional deletion of β-catenin in early progenitor cells using the Prx promoter revealed β-catenin is critical for osteoblast differentiation. These mice have significant skeletal abnormalities during development as osteochondral progenitor cells differentiate into chondrocytes instead of osteoblasts [139].

As the role of Wnt signaling is becoming inevitably important, the use of Wnt ligands to stimulate bone formation is becoming an intriguing idea. Of all the Wnt family members, Wnt10b has been the most well studied to induce bone formation [140]. Transgenic expression of Wnt10b in mice revealed a four-fold increase in trabecular bone volume and decrease in adipose tissue, indicating that this ligand enhances osteoblastogenesis while suppressing adipogenesis [140, 141]. Mice deficient in Wnt10b show a 30% reduction in trabecular bone volume with no difference in cortical bone volume or tartrate-resistant acid phosphatase (TRAP5b) serum levels indicating that Wnt10b seems to be an osteoblast specific molecule. Although most literature indicates Wnt signaling's importance in osteoblast differentiation, recent evidence indicates Canonical Wnt signaling inhibits osteoclastogenesis through Frizzled receptor 8 and Wnt3a [142]. There is also evidence that inhibition of the Wnt signaling pathway has an effect on bone remodeling.

The family of Wnt inhibitory factors (WIFs) include Secreted Frizzled-Related Proteins (SFRPs), Sclerostin (SOST), Connective Tissue Growth Factor (CTGF), and Dickkopf proteins (DKKS) [137]. Loss of SFRP-1 in mice enhances osteoblast differentiation and proliferation, indicating that SFRP Wnt antagonist regulates cells of the osteoblast lineage during bone remodeling [143]. Sclerostin is an osteocyte specific marker that plays a role in negatively regulating bone formation and stimulating bone resorption. Deletion of Sclerostin in mice under the SOST promoter revealed significantly more bone by µCT and DXA scanning in 16 week old male and female mice [144]. Furthermore, Sclerostin antibody treatment has shown to significantly increase bone formation, mass, and strength in a postmenopausal rat model [145]. These results indicate that both agonists and antagonists of the Wnt signaling pathway may potentially be used as a therapeutic to help treat bone disease in the case of osteoporosis.

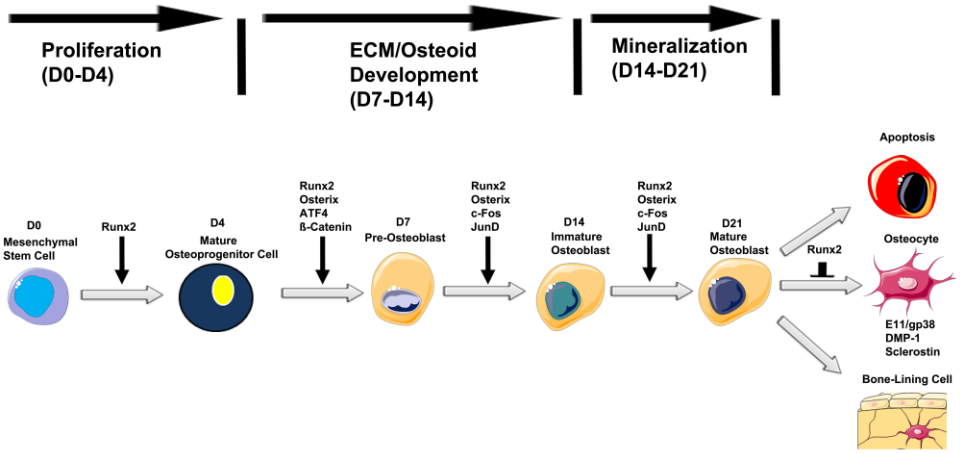
Parathyroid hormone (PTH) is an 84-amino acid single chain polypeptide hormone expressed primarily by the parathyroid glands. PTH and parathyroid hormone related peptide (PTHrP) have both anabolic and catabolic effects on bone metabolism. PTH secretion is regulated by several different mechanisms, including extracellular calcium levels, 1, 25$(OH)_2$ vitamin D, and Fibroblast Growth Factor 23 (FGF23) [146]. PTH signals through its 7-transmembrane receptor PTH1R and stimulates downstream PKA and PKC signaling which converge on the Runx2 and regulate osteoblastogenesis [147]. Mice deficient in PTH show a moderate phenotype with slightly large hypertropic zones in the growth plate [148]; however, mice deficient in parathyroid hormone related peptide (PTHrP) die perinatally, most likely due to abnormalities in endochondral

ossification [149]. Abnormalities in PTH signaling has been linked to the human disease Blomstrand Lethal Osteochondrodysplasia in which patients suffer from premature ossification and advancement of the skeleton due to a loss of function mutation in PTH1R [150]. There have been several mechanisms predicted of the role of PTH in enhancing bone formation. The effects of PTH and PTHrP seem to be context dependent. The actions of PTH on bone are cell type, dosage, exposure, and cell stage dependent. Previous literature indicates that PTH may have an effect on osteoblast proliferation, differentiation, and/or viability; however, PTH has also shown to play a role in osteoclast differentiation [151].

The Fibroblast Growth Factor (FGF) family of proteins has shown to play an important role in osteoblast differentiation and function. The FGF ligands bind to their corresponding tyrosine kinase receptors (FGFR) which dimerize upon ligand binding and stimulate downstream signaling pathways [152]. Several of the FGF ligands and their receptors (FGFR) are expressed during osteoblastogenesis. The FGF2 ligand (basic FGF) and its receptor (FGFR2) have been shown to be important modulators of bone development. Mice deficient in FGF2 at 4 weeks have significantly less trabecular bone by microCT, a lower mineral apposition rate (MAR), and less mineralization *in vitro* compared to controls [153]. Furthermore, FGFR2 homozygous mice die embryonically [154]; however, conditional mice deficient in FGFR2 reveal significantly impaired proliferation of osteoprogenitor cells and dysfunction of mature osteoblasts [155]. Dysfunction in the FGFR2 gene has been correlated with several human craniosynostosis disorders [156], indicating the importance of the FGF2 signaling pathway and osteoblasts. FGF2 administration in an overectomized rat model was

shown to restore bone mass [157], indicating the potential for FGF2 to be used as a therapeutic for treatment of osteoporosis. FGF2 downstream was also found to stimulate the MAPK/Runx2 pathway in osteoblasts [158]. Interestingly, the FGF pathway was found to interact with other molecules, such as integrins and heparin sulfate proteoglycans, to induce downstream signaling. The FGF3 ligand and receptor are also expressed in osteoblasts. FGFR3 deficient mice at a young age were shown to exhibit severe bone dysplasia and enhanced endochondral growth [159]; however, at an older age (16 weeks), FGFR3 deficient mice develop osteopenia and defective bone mineralization [160]. This indicates that the role of FGFR3 in bone remodeling is age dependent. FGF18 ligand plays a role in both osteoblast and chondrocyte development. Previous literature has shown that FGF18 deficient mice have delayed osteogenesis and increased proliferation and differentiation of chondrocytes [161]. This indicates that FGF18 is a positive regulator of bone formation and a negative regulator of chondrogenesis. Certain FGF receptor ligands have been shown to be important regulators for phosphate and vitamin D metabolism. FGF23 deficient mice have been shown to have significantly higher vitamin D and phosphate levels [162]. Furthermore, the authors concluded that FGF23 inhibits reabsorption of phosphate ions in the kidneys indicating a link between bone and kidney. This indicates that FGF23 is a key regulator in maintaining proper vitamin D and phosphate homeostasis. Several FGF ligands and their receptors have been shown to play a role in multiple systems including bone. Further research needs to be done to determine the role of other FGF ligands and their receptors in bone homeostasis.

There are several cellular and molecular determinants that have a diverse effect on osteoblasts (Figure 2.7). All of the pathways described have a major role on not only osteoblasts, but also a variety of cells within the bone microenvironment. The importance of determining the involvement of these molecules is crucial when determining new bone anabolic therapies to treat bone disease. The discovery of new anabolic factors that influence osteoblast differentiation and function independent or in-conjunction with these pathways is vital.



Known markers of osteoblast differentiation and their expression levels						
Runx2/Cbfa1	+	+	++	++	+	-
Osterix/Sp7	-	+	++	++	++	-
Alkaline Phosphatase (ALP)	-	+	++	++	+++	-
Col1α1	-	-	+++	+++	+	-
Osteocalcin (OCN)	-	-	-	-	+++	-
BMPs	+	++	+++	++	++	-
TGF-ß	+++	++	++	+	-	-
Wnts	-	+++	++	+	-	-
Parathyroid Hormone (PTH)	-	+	++	+	-	-
Fibroblast Growth Factors (FGFs)	-	++	++	++	+	-
α2-HS-glycoprotein	-	-	-	+	++	-
Decorin/Biglycan	-	-	++	++	-	-
Osteonectin	-	-	+	+	++	-
Bone Sialoprotein (BSP)	-	+	+	++	++	-
Matix Gla Protein	-	-	+	-	+++	+
Osteopontin	-	_	+	+	+++	+++

Figure 2.6: Schematic diagram depicting the different stages and known markers of osteoblast differentiation. Osteoblasts arise from mesenchymal stem cell precursors and require the critical transcription factors Runx2 and Osterix in order to differentiate into this lineage. In culture experiments, after a week, cells begin depositing an extracellular organic matrix (ECM) also known as osteoid. As the osteoblast begins to mature minerals are deposited which primarily consist of hydroxyapatite. Once these processes have ceased, the osteoblast has 3 potential fates. The osteoblast may undergo apoptosis, become an osteocyte; or enter a quiescent state and may lie flat on the bone surface and become bone lining cell. **-**=little to no expression; **+**=moderate expression; **++**=relatively high expression; **+++**=very high expression.

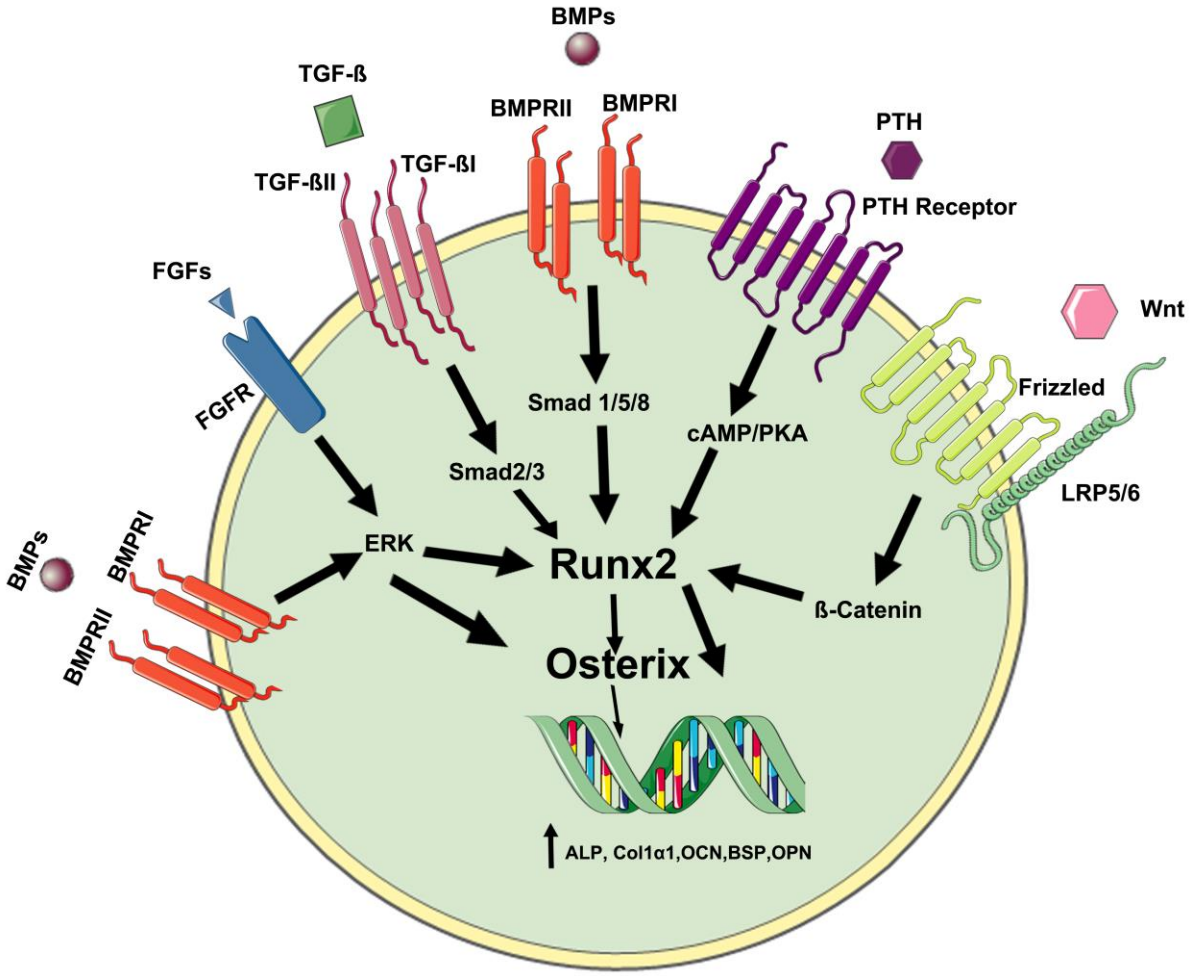


Figure 2.7: Schematic diagram indicating the important receptor-ligand interactions critical for osteoblast differentiation and function. Several signaling pathways have been found to be important for osteoblast differentiation and function. Runx2 is the critical transcription factor that most signaling pathways converge. Interestingly, Runx2 has been shown to regulate Osterix activity; however certain signaling pathways have been shown to act independently of Runx2 and mediate osteoblast differentiation and function through Osterix alone.

The process of bone remodeling requires both bone formation from the osteoblasts and bone resorption by the osteoclasts (Figure 2.8). In order to maintain proper bone homeostasis, bone formation and resorption acquire a balance between them. Once this balance is disrupted, pathological conditions within bone arise. In the more common pathological conditions, bone resorption by the osteoclasts exceeds bone formation leading to a reduction in bone mass. The osteoclast is a member of the monocyte/macrophage family arising from the hematopoietic stem cell lineage; it is found within the bone microenvironment that undergoes a series of differentiation processes to become a multinucleated cell [163]. The process of osteoclast differentiation is dependent on several cytokines within the bone microenvironment. The two essential cytokines for osteoclast differentiation are Receptor Activator of Nuclear Factor kappa-B ligand (RANKL) and Macrophage Colony Stimulating Factor (M-CSF; CSF-1) [164]. RANKL, also known as TNFSF11, is a member of the TNF superfamily and the critical cytokine involved in osteoclastogenesis due to its ability to direct precursor cells into the osteoclast lineage. M-CSF contributes to a series of events important for the proliferation, differentiation, and survival of the osteoclasts [165]. These two cytokines are both secreted and membrane bound, and are expressed by several other cells in the microenvironment, including osteoblasts, osteocytes, and immune regulator cells [166]. The RANKL and M-CSF ligands bind to their respective receptors, RANK and c-FMS, and activate signals to induce differentiation of osteoclasts. The series of events from osteoclast precursor to mature bone resorbing osteoclast require the involvement of several osteoclast-specific genes that are tightly coordinated and regulated.

Osteoclast formation is controlled by several factors and cells within the microenvironment. The osteoblasts are a major source of RANKL controlling osteoclast differentiation. The osteoblast also secretes a RANKL decoy receptor known as Osteoprotegerin (OPG), shown to inhibit RANKL induced osteoclastogenesis [167]. The RANKL/OPG axis is an important regulator of osteoclast differentiation. Previous literature has shown that OPG deficient mice develop early onset osteoporosis due to increased osteoclast activity [168]; therefore, when OPG levels are significantly reduced compared to RANKL, there is a significant increase in the number of osteoclasts. When OPG levels are significantly higher than RANKL, there is a significant reduction in the number of osteoclasts, indicating that the RANKL/OPG axis is an indicator of the relative number of osteoclasts. Although the RANKL/OPG axis is the key regulator of osteoclastogenesis, there are several other factors important for osteoclast differentiation.

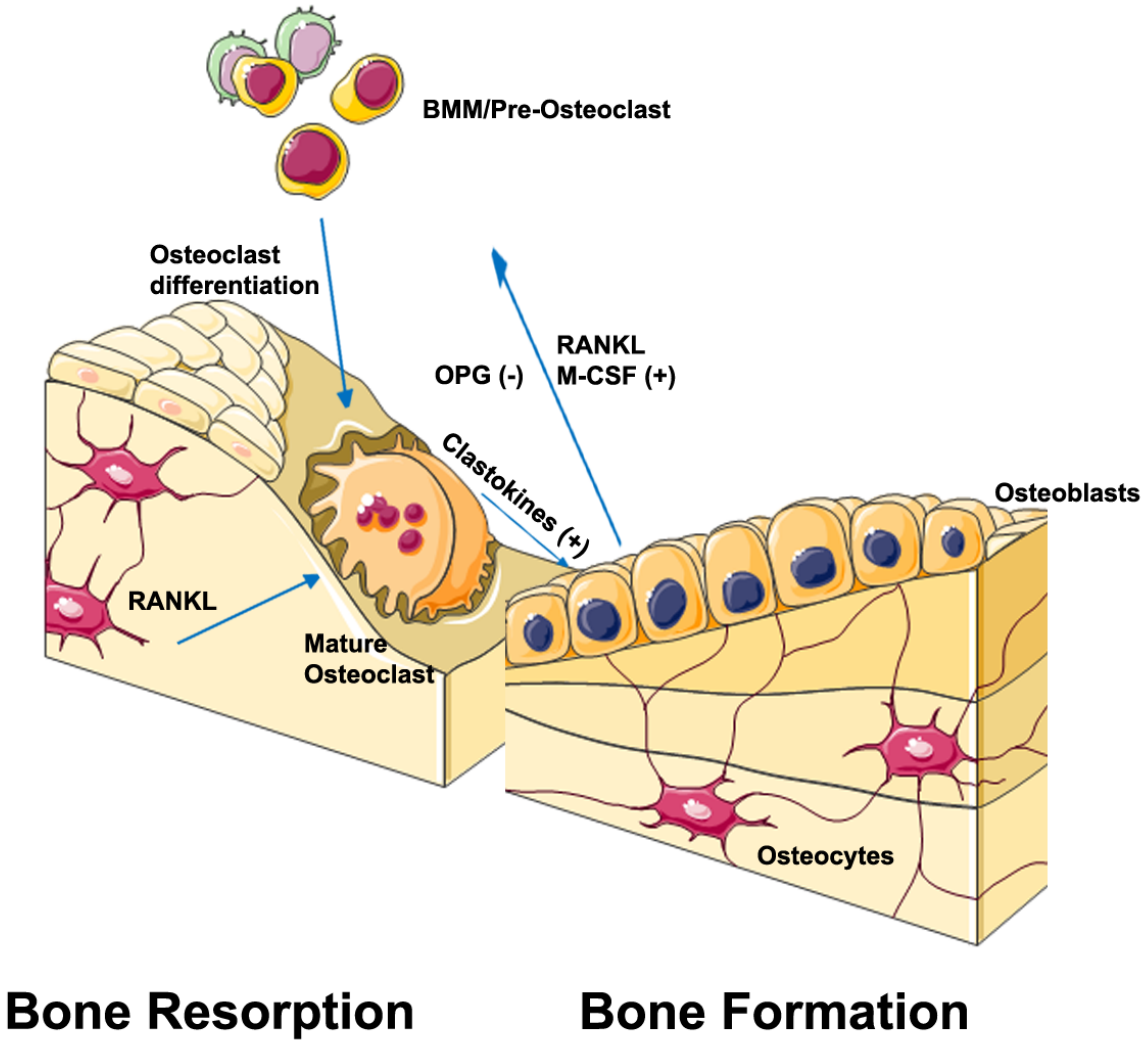


Figure 2.8: Summary of bone remodeling cycle. Osteoblasts differentiated from mesenchymal stem cell precursors secrete cytokines M-CSF and RANKL to stimulate osteoclast differentiation and bone resorption. Osteoblasts also secrete OPG, a negative regulator of osteoclast differentiation, to regulate RANKL activity. Osteocytes have also been shown to regulate osteoclast differentiation by secreting RANKL. Mature osteoclasts have been shown to secrete clastokines (molecules released by osteoclasts that affect osteoblasts) to regulate osteoblast differentiation and function.

There are several stages involved in the process of osteoclast differentiation, which include the proliferation, commitment, fusion, and activation. The earliest identifiable stage of the osteoclast is the granulocyte- macrophage colony forming cell (CFU-GM;BMM) which proliferate and differentiate to form a committed osteoclast precursor [169]. Early transcriptional regulation of osteoclast differentiation is controlled by PU.1 and MITF. PU.1 deficient mice have severe osteopetrosis due to a failure to generate bone marrow macrophages (BMM) [170]. PU.1 effects on early osteoclast differentiation are in part due to its interaction with the Microphthalmia (MITF) family of transcription factors. The lack of MITF ($MITF^{mi/mi}$) in mice leads to severe osteopetrosis due to the absence of mature osteoclasts; however, these mice are still able to generate macrophages, indicating that MITF is activated prior to PU.1 [171]. The M-CSF/c-FMS signaling axis is important for early stage osteoclast differentiation. The importance of M-CSF in osteoclastogenesis has risen from *op/op* mice that contain a mutation in the M-CSF gene leading that to a truncated version of the protein resulting in osteopetrosis due to lack of osteoclasts [172]. Interestingly, the skeletal phenotype of these mice resolves with age due to compensatory cytokines GM-CSF and VEGF [173, 174]. Previous literature has shown that M-CSF signaling regulates MITF and anti-apoptotic factor Bcl-2, indicating that M-CSF plays a role in both early commitment and apoptosis of osteoclasts [175, 176].

The final commitment stage from BMM to osteoclast precursor cell (OCP) is primarily controlled by RANKL/RANK signaling. The activation of RANK recruits members of the TNF Receptor-Associated Factor (TRAF) family of adaptor proteins [177]. The TRAF family contains seven members; however, the primary TRAF protein

associated with RANK signaling is TRAF6, which has three distinct binding domains to RANK [178]. Several groups have independently characterized the skeletal phenotype of TRAF6 deficient mice with slightly different results, but the accepted result is that these mice are severely osteopetrotic due to severe dysfunctional osteoclast differentiation [179-181]. The binding of TRAF6 to RANK leads to the activation of the NF-κB and MAPK pathways. The NF-κB pathway consists of a family of dimeric transcription factors that regulate several osteoclast specific genes. There are 5 NF-κB proteins which include Rel, RelA (p65), RelB, NF-κB1 (p50), and NF-κB2 (p52) [182]. In unstimulated cells these proteins remain in the cytoplasm; however, upon stimulation they translocate to the nucleus and regulate several different genes.

There are two different pathways of NF-κB activation: the classical and alternative. The classical, or canonical pathway involves activation of the IKK complex that phosphorylates and degrades IκB [183]. Unphosphorylated IκB binds to p50 and RelA and upon its degradation by the IKK complex, releases p50 and RelA, which translocate to the nucleus. In the alternative pathway, NF-κB-inducing kinase (NIK) stimulates the IKK complex to process p100 to release RelB to the nucleus, where along with p52, regulate a variety of genes [177]. Several of the proteins involved in the NF-κB pathway have resulted in an osteopetrotic phenotype due to impaired osteoclast differentiation [184-186]. The critical target gene of the NF-κB pathway is nuclear factor of activated T-cells-1 (NFATc1). The induction of NFATC1 is the hallmark event in the determination of BMM into osteoclasts, and its promoter contains NF-κB binding sites [187, 188]. NFATc1 binds to and regulates several osteoclast-specific genes, including TRAP [189], Calcitonin Receptor [190], Cathepsin K [191], β_3 integrin [192], and

OSCAR [189]. Activation of RANK in osteoclasts has not only shown to stimulate members of the NF-κB family, but also members of the MAPK family.

There are four groups within the MAPK pathway, which include p38 (α,β,γ,δ), JNK1/2/3, ERK1/2, and ERK 5 [193]. Previous literature has been documented explaining the importance of the MAPK pathway during osteoclastogenesis [194-196]. The MAPK pathway has shown to regulate the transcription factor complex AP-1 through the induction of its subunit c-Fos [197]. The AP-1 is a dynamic complex consisting of Fos (c-Fos, FosB, Fra-1, Fra-2,), Jun (c-Jun, JunB, JunD), and ATF (ATFa, ATF2, ATF3, ATF4, B-ATF) proteins [198]. Mice deficient in c-Fos are severely osteopetrotic due to lack of osteoclasts, indicating that this factor is an important regulator of osteoclast differentiation [199]. Interestingly, it has been shown that members of the AP-1 complex cooperate with members of the NF-κB pathway to regulate NFATc1 along with other osteoclast-related genes, indicating the importance of both pathways for osteoclastogenesis [188]. As shown, several regulatory mechanisms are required for the commitment of osteoclast precursor cells. Once the commitment into the osteoclast lineage is complete, the osteoclasts must bind to the bone surface, fuse, and begin the secretion of degradative enzymes.

Before the osteoclast begins its bone degradation process, it must first be able to attach to the bone surface. The recognition between the bone surface and the osteoclast cell membrane is controlled through integrins. Integrins are composed of αβ heterodimers that transduce signals between the extracellular matrix and the inner cytoplasm of the cell. The principle integrin mediating attachment in osteoclasts is $\alpha_v\beta_3$

[200]. Mice deficient in β_3 integrin have increased bone mass due to dysfunctional osteoclasts [201]. The integrin family members are able to recognize extracellular matrix proteins through the amino acid motif Arg-Gly-Asp (RGD). Several extracellular matrix proteins, such as Osteopontin and Bone Sialoprotein, have been shown to interact with $\alpha_v\beta_3$ allowing for cell adhesion and attachment to the bone surface [62].

The attachment of integrins within the osteoclast to extracellular matrix proteins results in the beginning steps of the formation of a sealing zone, one of the critical steps for bone resorption. In order for osteoclasts to resorb, they must first become polarized. The osteoclast cytoskeleton is a key mediator for its polarization and resorption. In the beginning stages of resorption, an acidified microenvironment is created by several components. Carbonic anhydrase II (CAII) and V-ATPase are the two main enzymes responsible for lowering the pH of the microenvironment by secreting H^+ and HCl. Furthermore, acidifed vesicles transported along microtubules within the cell containing degradative enzymes Cathepsin K and matrix metalloproteinases (MMPs) are carried to the plasmalemma and deposited onto bone matrix at what is known as the ruffled border [202]. The area between the ruffled border and the degraded bone matrix in which degradative enzymes and acidic molecules are being deposited is known as the Howship's lacunae [203].

Mutations in several osteoclast resorptive genes have been identified and correlated with human osteopetrosis. The degradation of bone matrix results in the release of minerals and collagen that accumulate within the blood stream. The increased concentration of minerals, such as calcium, triggers the secretion of specific hormones, such as calcitonin, to be expressed. Calcitonin binds to its receptor

(Calcitonin Receptor) which signals to the osteoclast to halt resorption [204]. Other hormones such as estrogen regulate osteoclast resorption by triggering apoptosis. Imbalance or loss of these factors can have severe consequences for bone homeostasis and lead to pathological conditions.

Although the RANKL/RANK signaling axis is the key regulatory factor for both osteoclast differentiation and function, several other inflammatory cytokines can contribute as well. The inflammatory cytokines of the tumor necrosis and interleukin families have shown to play a role in osteoclast differentiation and function. Previous literature has shown TNF-α, IL-1β, and IL-6 bind to their respective receptors TNFR and ILR and mediate downstream signaling [205, 206]. These factors along with RANKL can act as a synergistic effect during osteoclast differentiation and function.

The process of osteoclast differentiation and function is complex and regulated by several different cell types. Imbalance of the bone homeostatic mechanism between osteoclasts and other cell types can result in pathological conditions; therefore, the understanding of all aspects within the bone microenvironment and how they signal between each other is important for identifying new factors that may contribute to the process of bone remodeling.

Osteocytes comprise the greatest percentage of all bone cells (~90%) within the microenvironment [207, 208]. Osteocytes reside within the mineralized matrix of bone and form connections with each other and cells on the bone surface through dendritic processes [32]. The osteocyte cell body itself is encased within a structure known as the lacuna. Through their network of dendritic processes, osteocytes relay signals not only

to other osteocytes, but also to cells on the bone surface and within the bone marrow. The osteocytes arise from the mesenchymal stem cell and osteoblast lineages. One of the first proposals of osteocytogenesis stated that a subpopulation of osteoblasts become passively encased in osteoid which then becomes mineralized by neighboring osteoblasts [209, 210]. Other mature subpopulations of osteoblasts that do not under osteocytogenesis undergo apoptosis instead.

As a cell undergoes osteocytogenesis, the cell develops a polarity relative to the surface of the bone and develops dendritic processes that extend to other cells and the bone surface. Osteocytes express several cell specific markers. E11/gp38/Podoplanin is a marker expressed on the cell surface in embedded osteoid osteocytes [211]. Targeted deletion of this molecule in mature osteoblasts resulted in significantly fewer canaliculi per osteocyte and increased trabecular bone [209]. Several other osteocyte markers include PHEX, MEPE, DMP-1, FGF-23, Sclerostin, and ORP150. Osteocytes have been shown to have a large impact on bone remodeling through a variety of mechanisms which include regulation of the RANKL and Wnt/β-Catenin pathways and as sensors for biomechanical stimuli.

Osteocytes have been shown to secrete factors that stimulate both osteoclast and osteoblast differentiation. The number of healthy osteocytes and their ability to sense a variety of external stimuli can be correlated with the quality of bone. Osteocyte cell death can be associated with skeletal pathologies such as osteoporosis and osteoarthritis [212]. Osteocyte apoptosis can occur by several mechanisms, including microdamage, aging, glucocorticoid treatment, and estrogen withdrawal. In cases of microdamage to bone tissue, osteocyte apoptosis occurs, resulting in the release of

inflammatory cytokines within the microenvironment. Previous literature has shown that apoptotic osteocytes secrete large amounts of RANKL to recruit osteoclasts to the site of damage [213].

Prolonged use of glucocorticoids has been shown to inhibit bone formation, stimulate resorption, and cause osteonecrosis [212]. Glucocorticoid treatment in MLO-Y4 osteocyte-like cell line resulted in an increase in Fas/CD95 and Caspase 8 expression [214]. This mechanism may explain the link between prolonged use of glucocorticoids and osteocyte cell death. Estrogen withdrawal has been linked with an increase of proinflammatory cytokines. During post-menopausal periods, the lack of estrogen leads to an increase in TNF-alpha and interleukin-1 which have been shown to induce osteocyte apoptosis [215]. Several agents have been shown to reverse the effects of osteocyte cell apoptosis. These include Calcitonin, CD40 ligand, bisphosphonates, and estrogen modulators [209]. Interestingly, osteocytes also seem to be involved in osteoblast and MSC differentiation. The conditioned media collected from the MLO-Y4 cell line has been shown to stimulate MSC proliferation and differentiation [216]. This indicates that osteocytes may play a role in both bone formation and resorption.

One of the more well-known and primary functions of osteocytes is their ability to sense mechanical stimuli and regulate bone remodeling. The adult skeleton is able to adapt to mechanical loading by which new bone is added or removed based on the amount of stress placed on the tissue. Loading and unloading of bone tissue changes the gene expression in osteocytes *in vivo*, indicating that load effects osteocyte function [217]. In order to increase bone mass *in vivo* by mechanical strain, the ideal intensity,

frequency, and timing of loading must be within a certain range [218, 219]. One of the challenges that remain is to correlate what is known *in vivo* and translate it to *in vitro* parameters. Fluid-flow shear stress models are the gold standard in determining the role of osteocytes as mechnosensory cells. As mechanical strain is applied to bone, this causes a fluid flow through the canaliculi around the osteocyte resulting in shear stress and deformation of the cell membrane. The mechanical strain is sensed through the dendritic processes and cilia of the osteocyte [220]. In response to shear stress, osteocytes release NO, prostaglandin, and ATP [221, 222]. This mechanism may explain the effect of osteocyte mechanical loading on new bone formation *in vivo* and *in vitro*.

Osteocyte related proteins have shown to be important mediators of mineral metabolism. Osteocyte proteins DMP-1, PHEX, and FGF-23 have shown to regulate phosphate levels and biomineralization [223]. In the case of hypophosphatemic rickets, patients contain a mutation DMP-1 which regulates FGF-23 [66]. Under normal circumstances, DMP-1 down regulates FGF-23 allowing reabsorption of phosphate by the kidneys which in turn maintain appropriate phosphate levels for normal mineral homeostasis in bone. In the DMP-1 mutant condition, FGF-23 levels are high in osteocytes leading to phosphate excretion by the kidneys and impairment in biomineralization due to the lack of circulating phosphate. A similar condition with elevated FGF-23 levels is seen in patients with chronic kidney disease (CKD) [224]. Progression of CKD is correlated with increased levels of FGF-23. This indicates that osteocytes may act as an endocrine system that target distant tissue such as the kidney.

Osteocytes have multifunctional roles in bone remodeling. The maintenance and integrity of bone can in part be determined by osteocyte health, status, and viability. Osteocytes are involved in many pathological conditions including osteonecrosis, hyper/hypophosphatemia, and osteoporosis. Previous literature has shown that osteocytes may have a potential role as an endocrine organ in both the kidney and cardiovascular system [225]. Currently therapeutics to treat bone disease have been discovered from factors secreted by osteocytes. It will be interesting to determine if other factors expressed by osteocytes yet to be discovered will have an impact on bone remodeling.

2.4 Osteoporosis

As the longevity and quality of life improves in humans, age-related pathologies such as osteoporosis, have become a problem. Osteoporosis can be defined as a skeletal disorder in which bone integrity and strength are compromised, predisposing an individual to an increased risk of fracture [226]. In the United States in 2005, two million fragility fractures were reported and is predicted to increase to three million by 2025 unless more preventative measures take place [227]. Half of these fractures occur in patients with significantly low bone mass, and the other half occur in patients diagnosed with osteopenia.

The most common types of fractures seen in patients with osteoporosis include hip, vertebral, and distal forearm. The survival rates for patients five years after hip and vertebral fractures are roughly 80% compared to those men and women of similar age without fracture [228]. In terms of morbidity, 7% of survivors of all types of fracture have

in some instance permanent disability and 8% require nursing home care [229]. There is a broad use of terminology when describing osteoporosis. The terms used to describe cases of osteoporosis include postmenopausal, senile, and idiopathic [32]. The first two terms are synonymous, whereas idiopathic osteoporosis refers to premenopausal women and younger men. The pathogenesis of osteoporosis arises due to four basic mechanisms. The four basic mechanisms include accelerated bone loss due to increased osteoclast activity, inadequate bone formation by the osteoblasts, increased falls, and, most importantly, a failure to achieve optimal peak bone mass and strength [227]. These mechanisms are influenced by several factors, including genetics, nutrition, and lifestyle.

Genetics are the major determinant of peak bone mass and strength. As the individual ages, changes in gene expression occur that can eventually cause age-related pathologies such as osteoporosis. There are several important factors within the bone microenvironment that change overtime that will influence the bone remodeling process. Several genome-wide studies have been conducted to identify specific gene loci that regulate bone mineral density. The polymorphisms that occur within these loci are genes important for both bone formation and bone resorption. A few examples of polymorphisms that are correlated with osteoporosis and are important for osteoblast function are COL1A1, vitamin D receptor (VDR), and LRP5. Polymorphisms within the COL1A1 gene have been thought to be linked to skeletal fragility [230]. Multiple polymorphisms in the COL1A1 gene have been described [231, 232]. Changes in the COL1A1 gene effect critical functions of collagen within the bone matrix. For example, previous literature has shown that polymorphisms in COL1A1 result in an imbalance in α1 and α2 chains contributing to the impairment of the bone by reducing mineralization

resulting in decreased bone integrity and strength [233]. LRP5 is a co-receptor involved in the Wnt signaling pathway important for osteoblast differentiation and function. There are several studies that have recently been published showing evidence between polymorphisms in LRP5 and bone mineral density (BMD) [234, 235]. The mechanism in which the polymorphisms effect LRP function are not yet known; however, certain evidence has revealed that the polymorphisms in LRP5 effect its interaction with LRP6 [235]. VDR was one of the first candidates to be studied in relation to bone mineral density. Several polymorphisms in the VDR gene have been identified. One in particular polymorphism has been characterized within the promoter region that affects binding of the transcription factor Cdx-2 [236]. This polymorphism has been associated with BMD in Japanese subpopulations; however, in other subpopulations this polymorphism is associated with fracture but not BMD [237].

There are other polymorphisms linked to changes in BMD that are important for osteoclast differentiation and function. Previous literature has investigated the polymorphisms in oestrogen receptor α, TCIRG1, CLCN7, and Sclerostin [231]. One of the primary causes of postmenopausal osteoporosis is due to the lack of estrogen being produced. Estrogen regulates osteoclast viability and its withdrawal leads to a significant enhancement of bone resorption [238]; therefore, it can be expected that polymorphisms within the estrogen receptor would influence BMD in the aging adult. *TCIRG1* and *CLCN7* are both important mediators of osteoclast resorption. Inactivating mutations of either of these genes results in severe osteopetrosis [239, 240]. Currently there is little evidence to support any true correlation of polymorphisms within these genes and BMD. Sclerostin is an osteocyte specific marker that has been shown to

inhibit bone formation and stimulate bone resorption. SOST polymorphisms have been correlated with BMD in a study of both elderly men and women [241]. Future studies should emphasize the importance of the genetic basis of osteoporosis. This will increase the understanding of the mechanisms underlying the pathophysiology and help determine new markers to assess BMD and risk of fracture.

In addition to the importance of determining the genetic basis of osteoporosis, lifestyle choices can also be a determining factor for the onset of the disease. Physical activity, calcium, and vitamin D levels are primary determinants of bone mass and strength [242]. There are several environmental influences that may affect bone quality. These include smoking, excessive alcohol consumption, and immobilization [243]. Exercise and physical activity have shown to prevent and ameliorate osteoporosis in postmenopausal women [244]. Quality nutrition also has a large impact on the prevention of osteoporosis. Calcium and vitamin D intake are critical nutrients to prevent the onset of osteoporosis. During osteoporosis, increased osteoclast activity is seen due to a lack of calcium and vitamin D which leads to secondary hyperparathyroidism [242]. Previous literature has shown that calcium and vitamin D supplementation is not only important for the prevention of the disease, but can alsoactually reverse the effects [245]. Other nutritional benefits that can help alleviate the symptoms of the disease include protein intake, B vitamins, and vitamin K [246, 247]. Milk and dairy products along with fruit and vegetable consumption have been positively associated with bone health. It is necessary to incorporate all factors- genetic, environmental, and diet- when trying to prevent the onset of this disease.

2.5 Osteopetrosis

Osteopetrosis is a genetic disease characterized by a high bone mass phenotype due to a lack of bone resorption by osteoclasts [248]. It is a rare inherited disorder with an incidence of 1:250,000 effecting primarily cultures of Middle East and Northern European descent. There are two recognized forms of the disease. The autosomal dominant (benign) type, which has few symptoms, and the autosomal recessive infantile (malignant) type, which is typically fatal if not treated [249]. Common symptoms of the disease include an increase in BMD resulting in macrocephaly causing mental retardation, exophthalmos, micrognathia, and frontal bossing [250]. Secondary characteristics include low serum calcium levels resulting in hyperparathyroidism and neurological defects, including retinal atrophy and cranial nerve compression. Several mutations in osteoclast related genes important for differentiation and function that result in the osteopetrotic phenotype have been described [249, 251, 252]. In patients with osteopetrosis, there are two possible scenarios with respect to osteoclasts. In one instance osteoclasts are present but non-functional, and in the other they are relatively absent, although the mechanisms are different, the result is the same.

There are several mutations in osteoclast resorption genes that result in the osteopetrotic phenotype. Roughly 70% of the patients with this disease have mutations in either the TCIRG1 or CLCN7 gene [250] . The TCIRG1 gene encodes a 116 kDa isoform of the vacuolar (V)-ATPase proton pump, and CLCN7 (ClC-7) encodes the H^+-Cl^- exchange transporter 7 [239, 253, 254]. Both of these molecules are crucial for the formation of the ruffled border and lysosomal trafficking in osteoclasts. Other osteopetrotic genes related to dysfunction of the ruffled border include OSTM1,

PLEKHM1, and SNX10. OSTM1 encodes the β subunit of ClC-7 and prevents ClC-7 degradation by glycosylating the N-terminus [255]. OSTM1 is also thought to be important for Cl^- exchange [253]. PLEKHM1 is a 117 kDa cytosolic protein important for endosomal and lysosomal trafficking through Rab7 binding [256]. Mutations in PLEKHM1 lack the binding site for Rab7 resulting in an accumulation in the cytoplasm and an impairment of the osteoclast ruffled border. Mutations in the gene SNX-10 was only just recently found to be correlated with the osteopetrotic phenotype [257]. SNX-10 is a member of a family of proteins characterized by a PX domain, a phospholipid binding domain. The function of SNX-10 is still unclear; however, there is evidence that the PX domain within SNX-10 interacts with v-ATPase indicating that SNX-10 is an important regulator in subcellular trafficking [258].

Loss of function mutations involved in osteoclast differentiation can also lead to an osteopetrotic phenotype. Mutations within the RANK genes TNFRSF11A (RANK) and TNFSF11 (RANKL) result in a significantly reduced number of osteoclasts *in vivo* [259, 260]. Several of the characterized mutations within these genes have revealed that the mutation affects protein structure allowing inefficient binding of the respective proteins. Changes in protein structure are primarily due to either folding or improper glycosylation of RANKL [261]. Developing targets for RANK-RANKL interactions may potentially be used as a new treatment option for osteopetrosis.

Table 2.1 Summary of Described Molecules in Introduction: Function and Skeletal Phenotype of Mouse Models

Protein/Gene	**Animal Model**	**Function**	**Bone Phenotype**	**Reference**
α_2-HS-glycoprotein	Knockout	Regulates matrix mineralization	Ectopic calcification	33
Biglycan	Knockout	Positive regulator of bone formation; binds collagen and other growth factors	Osteopenic, short stature, decreased mineral content	36
Alkaline Phosphatase (ALP)	Knockout	Hydrolyzes mineral inhibitor pyrophosphates by increasing phosphate concentration	Decreased Mineralization; Impaired growth	40
Osteonectin	Knockout	Stimulates osteoblast proliferation and matrix	Severe osteopenia, decreased bone mineral content	42

		mineralization; Regulates osteoclasts		
Bone Sialoprotein (BSP)	Knockout	Stimulates osteoblast matrix mineralization; stimulates osteoclast differentiation	16 week animals have higher trabecular bone density, shorter femurs and body length	47,48
Osteopontin (OPN)	Knockout	Stimulates osteoclast resorption and osteoblast matrix mineralization	Develop normally; however ovarectomized mice are resistant to bone loss	50,51
Dentin Matrix Protein (DMP-1)	Knockout	Regulates osteoblast mineralization; important	Severe osteomalacia, abnormal tooth development	52, 53

		regulator of osteocyte function		
Fibrillin-1 and Fibrillin-2	Knockout	Regulate the bioavailability of other growth factors such as TGF-β and BMP-2	Fibrillin-1 knockouts are lethal;Fibrillin-2 have significantly less bone	60
Matrix Gla Protein	Knockout	Negative regulator of chondrocyte and bone mineralization	Lethal at 8 weeks due to calcification of vessels; premature growth plate mineralization resulting in less bone	63,64
Osteocalcin	Knockout	Believed to be transition signal from bone	Normal until 6 months and then develop osteopetrosis	69

		formation to bone turnover		
Runx2	Knockout	Critical transcription factor for osteoblast differentiation; regulates several important osteoblast and chondrocyte genes	Lethal; Completely lack osteoblasts and fail to form hypertrophic chondrocytes	85,87
Osterix	Knockout	Master transcription factor in osteoblasts; regulates several genes important for osteoblasts	Lethal; Complete absence of bone	89
LRP5	Knockout	Positive regulator of osteoblast	Significantly reduced	107

		differentiation and proliferation	trabecular bone volume	
LRP6	Knockout	Positive regulator of osteoblast differentiation and proliferation	LRP6 KO is lethal; but heterozygote is viable and reveals significantly less bone	110, 111
Sclerostin	Knockout	Negative regulator of bone formation and positive regulator of bone resorption	Deletion under the SOST promoter reveals significantly more bone by microCT	118
FGF2	Knockout	Positive regulator of bone formation	Global knockout is lethal; conditional knockouts have significantly less trabecular bone and impaired	127-129

			osteoprogenitor cells	
FGFR3	Knockout	Positive regulator of osteoblast differentiation	At 4 weeks develop bone dysplasia and 16 weeks are osteopenic due to defective osteoblast mineralization	133,134
FGF18	Knockout	Positive regulator of osteoblast differentiation and negative regulator of chondrogenesis	Decreased bone mass; increased chondrocyte differentiation	135
FGF23	Knockout	Essential regulator of phosphate and	Severe growth retardation and abnormal bone	136

		vitamin D metabolism	growth; short lifespan	
PU.1	Knockout	Positive regulator of osteoclast differentiation; transcription factor regulates several important osteoclast genes	Severe osteopetrosis due to inability to generate BMM	144
MITF$^{mi/mi}$	Knockout	Positive regulator of osteoclast differentiation; transcription factor regulates several important osteoclast genes	Severe osteopetrosis due to inability to generate mature osteoclasts	145
op/op	Mutant	Positive regulator of osteoclasts	Osteopetrotic due to lack of osteoclasts resulting from a	146

			mutation in the M-CSF gene	
TRAF6	Knockout	Positive regulator of osteoclast differentiation and function; Subunit of RANK receptor	Osteopetrotic due to dysfunctional osteoclast differentiation	153-155
β3 Integrin	Knockout	Positive regulator of osteoclast resorption and ruffled border formation	Osteosclerotic and osteopetrotic due to dysfunctional osteoclast resorption	175
TCIRG1	Mutant	Positive regulator of osteoclast resorption; Subunit of proton pump	Severely osteopetrotic due to lack of osteoclast resorption	211
CLCN7	Mutant	Positive regulator of osteoclast resorption;	Severely osteopetrotic due to lack of	225,226

		Important for H+/Cl- Exchange	osteoclast resorption	
TNFRSF11A (RANK)	Mutant	Positive regulator of osteoclast differentiation and resorption	Severely osteopetrotic due to lack of osteoclast number and resorption	231
TNFSF11 (RANKL)	Mutant	Positive regulator of osteoclast differentiation and resorption	Severely osteopetrotic due to lack of osteoclast number and resorption	232,233
Caveolin-1	Knockout	Believed to play a role in regulating both osteoblast and osteoclast differentiation	Increased bone size and stiffness; increased bone mass at 5 and 8 weeks	73

CD44$^{-/-}$-hTNF-Tg	Mutant	Plays an important role as an anti-inflammatory receptor	Mice have severe arthritis and are osteopenic due to high levels of inflammation	362

2.6 Bone Fracture

The unique structure of bone tissue allows it to withstand harsh mechanical forces and external physical stimuli. Unfortunately, in some instances bone tissue is unable to withstand the mechanical strain of certain forces, and a fracture develops. Fractures can be caused by several reasons, but the two most common are increased bone fragility due to age, or pathological condition, and severe skeletal trauma. There is a positive correlation with age and the potential risk of fracture. There have been several models proposed to assess the potential risk of fracture, but the two most common indicators are volumetric bone mineral density (vBMD) and cortical thickness measured by quantitative computed tomography (QCT), or high-resolution peripheral QCT (HRpQCT) [262]. The types of fractures can be classified based on several different criteria.

Fracture classification is complex; however, a set of guidelines designed by the Orthopaedic Trauma Association (OTA) and the OA pediatric group have attempted to unite a common diagnosis [263]. These guidelines include several factors, such as the

type of bone, location, morphology, and severity. All of these criteria are important for the prognosis and treatment options. In Muller's AO classification of fractures for long bones, in each long bone the regions of the bone are named and numbered (proximal, diaphyseal, distal) [264]. Based on the type of bone, what segment the fracture is located, and the fracture morphology determine the type of fracture. In long bones the section is first identified (proximal, diaphyseal, or distal), followed by the severity and morphology (commonly referred to as simple, partial/wedge, and complete/complex in order of severity). This is a general explanation: fractures can be further subdivided and there are also exceptions. Based on these criteria clinicians can make decisions for treatment options. Treatment options can vary from casting to surgical intervention. Understanding the underlying mechanisms responsible for skeletal healing will allow for the discovery of new treatment options.

The process of skeletal repair is critical for reconstitution of bone integrity after trauma or surgical intervention. Skeletal repair at the cellular level requires two distinct processes called intramembranous and endochondral ossification [265]. During intramembranous ossification a foundation of MSCs becomes vascularized and differentiates into mature matrix secreting osteoblasts [266]. This type of development is seen in flat bones such as the scapula and craniofacial bones and has no cartilage foundation. During endochondral ossification a cartilaginous template is developed first followed by vascular invasion, MSC differentiation into mature matrix secreting osteoblasts, and remodeling by resorptive osteoclasts. Within the fracture site endochondral bone formation typically takes place closest to the fracture site due to low oxygen tension and vascular disruption, whereas intramembranous bone formation

occurs distal to the site where vasculature is still present. The primary source of osteoprogenitor cells during fracture repair comes from the periosteum. The periosteum contains two distinct layers: an outer fibrous layer containing fibroblasts that connect to underlying cortical bone and an inner cambium layer consisting of multiple MSCs and osteoprogenitor cells responsible for healing [267]. During fracture repair a unique series of events takes place to repair the defect. Immediately following trauma vascularization is disrupted and a hematoma begins to form. Progenitor cells within the periosteum are recruited to the fracture site. In areas of low oxygen tension MSCs begin to differentiate into chondrocytes, and in areas where oxygen tension and vascularizton are normal, MSCs directly differentiate into osteoblasts and begin forming new bone [268]. Endochondral formation continues as chondrocytes begin to mature and mineralize, while simultaneously vascularization begins to ensue at the fracture site. With the emergence of a new vascular system, MSCs migrate to the area and begin to differentiate into osteoblasts and form a callus at the injured site [32]. The process proceeds as osteoblasts and osteoclasts remodel the newly formed woven bone into organized lamellar bone until anatomical restructuring is complete. The amount of time this process takes varies based on the severity of the fracture. There are also genetic and environmental factors that may influence this process.

The skeletal repair process involves multiple cell types and therefore involves multiple genetic determinants. Growth factors and cytokines important for the regulation of osteoblast differentiation and function are important mediators for skeletal repair. These mediators include BMP-2, TGF-β, Wnt, FGF, and IGF family of proteins [269]. BMP-2 is one of the more notable factors involved in skeletal healing as expression is

high in early callus formation just slightly after the time of fracture [270]. Previous literature has also shown that elimination of BMP-2 in limb buds disrupts fracture healing, indicating the importance of BMP-2 in the repair process [271]. The presence of angiogenic factors for vascularization at the fracture site is also important for healing. Vascular endothelial growth factor (VEGF) and its receptor (VEGFR) are upregulated during the repair process. Exogenous administration of VEGF in a mouse fracture model accelerates vascular growth into the callus and promotes bone formation in the area [272]. The accumulation of VEGF at the fracture site is thought to be in response to BMP-2, indicating a direct link between both osteogenic and angiogenic factors in the repair process.

Inflammatory cytokines important for bone turnover are important for skeletal healing. During the stage in which cartilage begins to mineralize, a turnover stage occurs that requires the upregulation of RANKL, M-CSF, OPG, and TNF-α [273]. The inflammatory cytokines IL-1 and IL-6 are also upregulated during the remodeling process and are believed to be important factors for osteoclast recruitment [274]. Cox-2, an enzyme responsible for catalyzing prostaglandins into arachidonic acid, has also shown to play a role in the repair process. Previous literature has shown that $Cox2^{-/-}$ mice have delayed bone repair compared to wildtype [275]. Histological analysis revealed delayed chondrogenesis from the fracture callus, indicating this molecule is an important mediator of chondrogenesis during fracture repair. Future studies should focus on determining other important factors involved in all phases of skeletal repair.

2.7 Current and Future Therapies

The prevention and treatment of osteoporosis requires several different approaches that include changes in lifestyle, diet, and pharmacological intervention. Exercise and maintaining appropriate muscle tone is often overlooked as a method of preventing the onset of osteoporosis. Several studies have shown that bone structure is enhanced by exercise and is compromised by immobility [276]. During the aging process, bone loss is inevitable and exercise through mechanical strain can counteract the effects of bone loss. Several studies have shown that most people, especially women, do not acquire the recommended levels of calcium and vitamin D in their diet [277, 278]. The recommended calcium and vitamin D intake from the U.S. National Academy of Sciences (NAS) varies based on age. The recommended intakes of calcium for ages 51+ are 1,200 mg/d, whereas vitamin D levels for this same age are approximately 10 µg/d [279]. The consumption of a proper diet in combination with a daily supplemental regiment can aid in acquiring the appropriate calcium and vitamin D levels. There have been several pharmacological compounds developed to help treat osteoporosis within the past few decades. These compounds can be categorized based on their target. These two categories include the anti-resorptive drugs (inhibit osteoclast function) and the anabolic agents (stimulate bone formation).

Anti-resorptive therapies are the most commonly used pharmacological compounds used to treat osteoporosis. Hormone therapy is the earliest recognized treatment for osteoporosis. Estrogen is important for maintaining normal bone homeostasis by acting on both osteoblasts and osteoclasts. Estrogen and its receptor are expressed in osteoblasts and play a role in enhancing the proliferation and

differentiation while inhibiting apoptosis in these cells [280, 281]. Previous literature has shown that estrogen treatment given at the onset of menopause completely prevents bone loss and fractures in women [282]. Estrogen treatment in women postmenopausal over a period of ten years revealed an increase in bone density of the spine and a reduction in spine and hip fracture by 33% [283, 284]. There are, however, several adverse events when using estrogen therapy. There have been several cases of endometrial carcinoma, thromboembolisms, stroke, and myocardial infarction [284]. These adverse events have led to the development of a new class of drugs for the treatment of osteoporosis known as selective estrogen receptor modulators (SERMs). These compounds have a mixture of antagonistic and agonistic functions properties based on tissue type [285]. The first SERM that was shown to have positive effects on bone mass was tamoxifen, used in the treatment of breast cancer [286]. Since then several other SERM therapies have been developed to help combat osteoporosis. Raloxifene has also shown to have effects with reducing bone resorption and has been shown to reduce the incidence of vertebral fractures [287]. This drug, however, has been linked to fatal strokes and thromboembelisms [288]. Currently there are a few other SERMs in clinical trials for the treatment of osteoporosis. Clinical data on basedoxifene and lasofoxifene are currently being obtained to determine their effects on bone mass [289]. These drugs have comparable effects to those seen in Raloxifene. Future studies need to focus on identifying other SERMS that stimulate bone mass to help treat osteoporosis.

The bisphosphonate compounds have been the most commonly used anti-resorptive drug for the treatment of osteoporosis. Bisphosphonates consist of a central

carbon atom with two adjacent negatively-charged phosphate groups which allows for high affinity for positively-charged hydroxyapatite in the bone matrix [284]. Bisphosphonates accumulate on the bone surface and become ingested within osteoclasts. Once accumulated within the osteoclast, these drugs interfere with metabolic pathways important for osteoclast function. The primary target is the mevalonate pathway in which the farnesyl pyrophosphate synthase (FPPS) enzyme that regulates cholesterol and isoprenoid metabolism necessary for prenylation of GTPases such as Ras, Rab, and Rho important for the osteoclast cytoskeleton, is inhbited [290]. Impairment of the cytoskeleton effects osteoclast attachment to the bone resulting in increased apoptosis. Currently there are several Bisphosphonates prescribed for the treatment of osteoporosis that include alendronate (Fosamax®), risedronate (Actonel®), zoledronic acid (Reclast®), ibandronate (Boniva®), and etidronate (Didronel®) [291]. Bisphosphonates have shown to rapidly reduce bone resorption and balance the increase in osteoclast activity due to the onset of menopause. They have shown to significantly reduce the risk of vertebral fractures [292, 293] and non-vertebral fractures [294-296]. Bisphosphonates have also been shown to increase bone mineral density in the spine and hip compared to placebo [297]. There are, however, several adverse side effects related to bisphosphonate treatment. The two most common side effects reported are gastrointestinal inflammation [298] and osteonecrosis of the jaw [299].

There are other anti-resorptive compounds that have potenitial use for the treatment of osteoporosis. Denosumab (Prolia®) is a RANKL antibody approved for the treatment of osteoporosis by subcutaneous injection. Previous literature has shown a 90% reduction in osteoclast biomarkers and comparable results to those seen by

bisphosphonates. The adverse side effects include muscle pain, hypocalcemia, and increased risk of infection [300]. Inhibitors against the osteoclast degredative enzyme Cathepsin K have also shown some potential. Odanacatib® is a Cathepsin K inhibitor developed by Merck that is under clinical trials. In a two year study Odanacatib was shown to increase spine and hip bone mineral density [301, 302]; however, predicted side effects include stroke, atrial fibrillation, and atypical fractures.

Although the primary source of treatments are anti-resorptive and affect osteoclasts, there are few anabolic factors that affect osteoblasts. Currently teriparatide (Forteo®) [PTH(1-34)] is the only anabolic drug to treat osteoporosis**.** In a world-wide study of postmenopausal women, PTH injections showed a 13% and 6% increase in lumbar spine BMD (LS-BMD) and femoral neck BMD (FN-BMD) respectively [303]. The drug also revealed a 69% and 54% decrease in vertebral and non-vertebral fractures respectively. Another study showed that PTH is able to increase cortical thickness and cross-sectional bone area [304]. Although PTH has several benefits for increasing BMD, there are a few adverse effects. One of the more severe side effects reported in long-term PTH use is osteosarcoma [305]. Since PTH affects both osteoblasts and osteoclasts, long-term use can actually stimulate osteoclasts and result in hypercalcemia; therefore, PTH treatment needs to be monitored to determine appropriate dosing and timing. Although PTH is the only FDA approved anabolic agent for the treatment of osteoporosis, there are other compounds currently in clinical trials.

Strontium ranelate has been thought to be a perfect candidate as it shows both antiresorptive and anabolic responses [306]. In clinical trial strontium ranelate taken daily decreased the risk of vertebral fractures in postmenopausal women by 40% [307].

Strontium renelate is currently available in Europe but not in the U.S. due to the adverse effects of heart attack and thrombosis noticed in patients. An exciting new therapy that is being developed is the use of anti-sclerostin antibodies for the treatment of osteoporosis. Currently the anti-sclerostin antibody romosozumab is under phase II clinical trials [308]. The drug is interesting as it has both anabolic and anti-resorptive functions in bone by stimulating the Wnt pathway. With the emergence of new anabolic agents coming into clinical trials, it will be interesting to see what new therapies will emerge.

Treatments provided for patients with osteopetrosis can vary greatly based on the symptoms and mutation of the specific gene. The most common and successful treatment option for osteopetrosis is hematopoietic stem cell transplants (HSTC). The use of HSTC for treating the osteopetrotic phenotype came from the use of animal models in which bone marrow or splenic cells were transplanted from a healthy donor into the osteopetrotic mouse [309]. The first successful human transplant using HLA-identical marrow along with total body irradiation and cyclophosphamide therapy was reported a few years later [310]. Studies suggest that patients who receive HLA-identical HSCT have an estimated 88% chance of having a five year disease-free state [311]. Although HSCT transplants have a high success rate, there are a few caveats with this treatment option. Certain types of osteopetrosis based on the mutation contain neurodegenerative symptoms that cannot be reversed with HSCT treatment. With the use of HSCT, there is also the possibility of rejection; therefore, new and alternative treatment options need to be developed for those specific conditions.

Other alternative methods include the use of gene therapy, *In utero* HSCT, and treatments exploiting the RANKL pathway. Genetically modifying HSCs through the use of gene therapy could serve as a viable option as obtaining CD34+ cells is less invasive than transplantation. Previous literature has shown the efficacy of gene therapy through the use of animal models in which HSCs were programmed to overexpress TCIRG1 through retroviral expression [312]. These osteopetrotic mice showed improvement in bone resorption; however, the use of oncogenic viral vectors raises serious concerns for secondary adverse effects. *In utero* HSCT provides a viable treatment option as patients who undergo transplantation earlier have much better outcomes. Animal models using *In utero* HSCT in TCIRG1 deficient mice showed complete rescue in 35% of the implanted mice [313]. The caveats for using *In Utero* treatments are the use of reliable genetic tests to determine if the disease will be present along with the probability that neurological symptoms related to osteopetrosis will still remain unalleviated. The use of RANKL systemically to help restore osteoclast activity is also being examined as an alternative treatment option. Previous literature has shown that RANKL administration in TNFSF11 (RANKL) deficient mice at the correct dose and time rescued the bone phenotype [314]. Interestingly, RANKL administration was able to improve function of other haematopoietic organs, such as the thymus and spleen. Determining RANKL dose and timing in humans will be a challenge as overuse of RANKL will lead to a variety of adverse events.

Current treatments of severe trauma rely on bone grafting procedures. Historically, harvesting iliac crest bone graft (ICBG) has been the gold standard for treating massive bone grafting procedures; however, ICBG harvest is limited by the

amount of graft available as well as a relatively high complication rate. The discovery of potential molecules to enhance bone formation following skeletal trauma is crucial.

Currently, the only FDA treatment approved therapy is recombinant BMP-2 (rhBMP-2) marketed by Medtronic as INFUSE® [315]. INFUSE combines the rhBMP-2 with an absorbable collagen sponge and is approved for interbody spinal fusions in clinical use with the potential for tibial fractures and craniofacial bone repair in future applications. Previous literature has shown a number of positive outcomes with the use of INFUSE® as a bone graft substitute [316-318]. Recently, however, there is evidence of adverse events caused by INFUSE®, including overgrowth of bone, inflammation, edema, neurological impairment, and carcinogenicity [319]. The clinical and cost effectiveness of INFUSE® has also been a subject of much debate [320]. Although PTH has been approved only for the treatment for osteoporosis, the idea of its use to accelerate skeletal healing has also been tested. Recent evidence has revealed a positive effect for bone regeneration in a fracture model [321]. PTH treatment has shown to stimulate both chondrogenesis and MSC proliferation through upregulation of Sox9 indicating that PTH may play a role in the early process of skeletal healing [322].

Table 2.2 Summary of Pharmaceutical Compounds used to treat bone disease

Name	Brand Name	Class	Mechanism	Progress
Estrogen	Premarin®, Estratab®	Anti-resorptive	Stimulates apoptosis of osteoclasts	Available on market
Selective estrogen receptor modulators (SERM)	Tamoxifen®, Raloxifene®, Basedoxifene®, Lasofoxifene®	Anti-resorptive	Estrogen receptor agonist	Some currently available others in clinical trials
Bisphosphonates	Fosamax®, Actonel®, Reclast®, Boniva®, Didronel®	Anti-resorptive	Target mevalonate pathway;inhibit GTPases important for osteoclast cytoskeleton	Available on the market
Denosumab	Prolia®	Anti-resorptive	RANKL antibody	Available on the market
Odanacatib	MK-0822	Anti-resorptive	Cathepsin K inhibitor	Phase III clinical trials

Teriparatide (PTH 1-34)	Forteo®	Anabolic	Stimulate both osteoblast and osteoclast differentiation; sensitive based on dosage and timing	Available on the market
Strontium renelate	Protelos	Anabolic	Enhances collagen production	Approved in Europe; not approved in U.S. due to cardiac complications
Romosozumab	Marketed by Amgen	Anabolic and Anti-resorptive	Sclerostin antibody	Phase II clinical trials
rhBMP-2 bone graft	Infuse®	Anabolic	Recombinant BMP-2 stimulates osteoblast differentiation	Available on the market

2.8 Osteoactivin

As described previously, with the aging population growing, the search for new therapeutics is at its peak. Through the use of genetically manipulated animals to mimic disease state, new molecules important for bone development have been discovered. Osteoactivin (OA) was first discovered in a model of the *op* osteopetrotic rat [1]. Osteoactivin, also known as Glycoprotein Non-Metastatic Melanoma Protein B (GPNMB), is located on chromosome 7p15 and has high homology with other proteins, including Dendritic Cell Heparin Sulfate Proteoglycan Integrin Dependent Ligand (DC-HIL;88% homology), Human Hematopoietic Growth Factor Inducible Neurokinin (HGFIN; 77%), and Pmel-17/gp100 melanocyte protein (60%) [3]. The Osteoactivin cDNA has an open reading frame of 1,716 base pairs and encodes a 115 kDa type transmembrane protein (~572 amino acids) that is heavily glycosylated with several functional domains. Bioinformatic analysis of the Osteoactivin protein has identified a signal peptide (1-23), a polycystic kidney disease domain (PKD; 263-319), a proline-rich repeat domain (PRRD; 320-345), a transmembrane domain (500-521) and a dileucine signal on the C-terminal domain (Figure 2.9) [3, 323]. The protein is heavily glycosylated with a predicted 11 *N*-linked and 19 *O*-linked glycosylation sites. In order to understand potential functional roles of Osteoactivin, it is important to know how these domains and glycosylation sites correlate with cell structure and function.

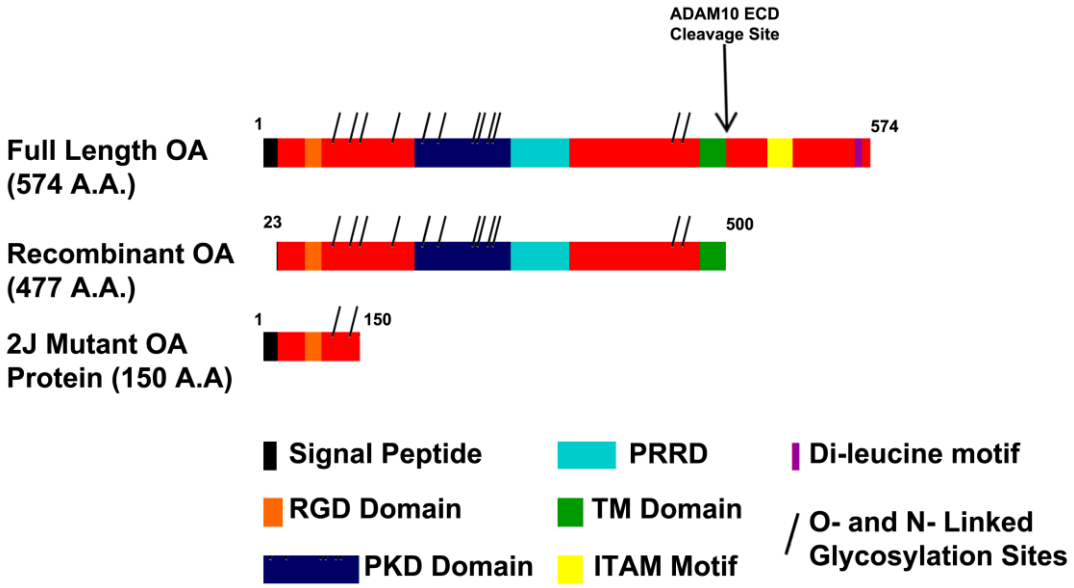


Figure 2.9: Diagram depicting the domains and glycosylation sites of full length, recombinant, and mutant Osteoactivin (OA). The full length Osteoactivin is 574 amino acids and has several different domains and glycosylation sites. The N-terminal extracellular domain of Osteoactivin (1-502) contains a signal peptide (1-22), and RGD domain, a polycystic kidney disease domain (PKD; 256-316), a proline rich repeat domain (PRRD; 320-345), and several O- and N- linked glycosylation sites. The transmembrane domain of Osteoactivin is 20 amino acids (503-523). The C-terminal intracellular domain (524-547) contains an hemITAM motif and Di-leucine signal [324]. The recombinant Osteoactivin represents the N-terminal portion of the molecule (23-502). It is derived from a mouse myeloma cell line and contains all of the same extracellular domains and glycosylation sites as the full length protein.

Osteoactivin and its homologs have been described in several different tissues. Following the discovery of Osteoactivin in bone, a previous report showed that certain bone-related genes are expressed in advanced malignancies and induce cancer metastasis and invasion [325]. This group revealed that bone related genes Osteonectin and Osteoactivin are highly expressed in a glioma human cancer model, and induce metastasis through increased MMP production, specifically MMP-3 and MMP-9. This indicates that Osteoactivin plays a role in cancer metastasis. Furthermore, another previous report has shown that Osteoactivin is highly expressed in breast cancer cells that metastasize to bone [326]. This group developed a model in which breast cancer cells overexpressing Osteoactivin enhanced the formation of osteolytic bone metastasis *in vivo*. The mechanism involved in Osteoactivin induced breast cancer tumor growth and metastasis is believed to involve an interaction with neuropilin-1 and $\alpha_5\beta_1$ [327]. Since the discovery of Osteoactivin and its role in breast cancer metastasis, several other groups have published the role of Osteoactivin in other cancer tissues, including prostate [328], pancreas [329], liver [330, 331], lung [332], melanoma [333-335], brain [336], and colon [337]. This indicates that Osteoactivin may serve as a potential target for treating several different types of cancers. Currently, there is an Osteoactivin antibody-drug conjugate (Glembatumumab Vedotin) in phase II clinical trials for the treatment of advanced and local breast cancer and melanoma [338-340]. Although Osteoactivin has been shown to act as a potential oncogene, Osteoactivin has also shown to play a vital role in a variety of processes important for cellular function.

Osteoactivin has shown to have a role as an anti-inflammatory agent in several different tissue types. Previous reports have shown that expression of Osteoactivin is high in response to injury seen in the liver [341, 342], muscle [343, 344], kidney [345, 346], and brain [347-349]. In response to injury within the liver, Osteoactivin expression was immediately high and was correlated with accumulation of inflammatory cells [341]. Interestingly, treatment with lipopolysaccharide (LPS), a known toxin, decreased Osteoactivin expression. This indicates the importance of Osteoactivin as an anti-inflammatory molecule. Furthermore, transgenic overexpression of Osteoactivin has been shown to attenuate liver fibrosis in a rat model [342]. In muscle, Osteoactivin expression was shown to be high in cases of denervated skeletal muscle [343]. In this study Osteoactivin was shown to increase the infiltration of fibroblasts into the area and increase matrix remodeling genes MMP-3, MMP-9, and type I collagen. This indicates that Osteoactivin may play a role in the regulation of matrix remodeling.

Osteoactivin has also been shown to act as a neuroprotective factor in neurodegenerative disease such as Amyotrophic Lateral Sclerosis (ALS) [348]. In this study Osteoactivin extracellular fragments were shown to rescue motor neurons by attenuating cell toxicity by superoxide dismutase-1. Another group showed that Osteoactivin acts as a neuroprotective factor in cases of cerebral ischemia reperfusion injury through an AKT and ERK mechanism [349]. The role of Osteoactivin in tissue repair may best be explained through the correlation of Osteoactivin expression and the accumulation of immune regulatory macrophages. A previous study revealed that Osteoactivin overexpression in the

monocyte/macrophage RAW264.7 cell line resulted in the reduction of inflammatory cytokines IL-6 IL-12p40 and NO production [350]. Furthermore, Osteoactivin was shown to play a role as a phagocytic protein important for phagocytosis of cellular debris in a renal injury model [351]. These data indicate that Osteoactivin has a large role in response to tissue injury. Based on these findings Osteoactivin may have a dual role as an anti-inflammatory molecule and as an oncogene.

Osteoactivin has been shown to be highly expressed in bone tissue. Initial studies of Osteoactivin and bone were discovered in osteoblasts [1, 352], and expression was shown to increase over the course of differentiation. The importance of Osteoactivin during osteoblast differentiation was first shown through the use of Osteoactivin antibodies. Osteoactivin antibodies added in culture resulted in an inhibition in osteoblast differentiation *in vitro* by ALP staining and activity and mineralization [2]. Furthermore, Osteoactivin overexpression *in vitro* in both MC3T3-E1 and C2C12 cell lines resulted in enhanced osteoblast differentiation [3, 4]. These reports indicate that Osteoactivin plays an important role in osteoblast differentiation. As previously mentioned, Osteoactivin has shown to increase during osteoblast differentiation. Previous reports from our lab have shown that BMP-2 regulates Osteoactivin by increasing its expression through homeodomain transcription factors DLX-3, DLX-5, and MSX-2 during osteoblast differentiation [353, 354]. Further evidence of the importance of Osteoactivin comes from the use of animal models *in vivo*. Overexpression of Osteoactivin under the CMV promoter (OA-Tg) in mice resulted in increased bone mass *in vivo* and *ex vivo* osteoblast differentiation [5]. A previous study generated an Osteoactivin mutant model that contains a missense

mutation in the Osteoactivin gene resulting in a truncated 150 amino acid protein. These mice (D2J) were shown to have early onset pigmentary glaucoma and hearing loss [355, 356]. Our lab was the first to characterize the bone phenotype in the D2J mice. D2J mice have an intrinsic impairment of osteoblast differentiation both *in vitro* and *in vivo* [6]. D2J have reduced bone mass by microCT and histology *in vivo* and dysfunctional osteoblastogenesis *ex vivo*. Furthermore, D2J osteoblasts were shown to have higher levels of TGF-β receptor which may explain the inhibition of osteoblast differentiation. Osteoactivin has shown to play an important role in osteoblasts; however, Osteoactivin also plays an important role in osteoclasts.

The role of Osteoactivin and osteoclasts was first determined *in vitro* through the use of anti-Osteoactivin antibodies. This group revealed that Osteoactivin increases during osteoclastogenesis, and that addition of anti-Osteoactivin antibodies inhibits osteoclast differentiation through its interaction with β_1 and β_3 integrin [8]. Furthermore, this group showed that overexpression of Osteoactivin in tissue specific mice using the TRAPC promoter resulted in less bone mass *in vivo* due to enhanced osteoclast differentiation and resorption [9]. Interestingly, another group recently published that the Osteoactivin mediated increased resorption is initiated through binding of $\alpha_v\beta_3$ integrin [10]. Our group has also examined the effects of Osteoactivin using D2J mice. Interestingly we found D2J have less trabecular bone *in vivo* but thicker cortical bone and narrow medullary areas [7]. This indicates that there is impairment in both osteoblasts and osteoclasts due to lack of functional Osteoactivin. Osteoactivin plays different roles in osteoclast differentiation and function. D2J osteoclasts *ex vivo* differentiate more and are larger in size indicating

that Osteoactivin negatively regulates osteoclast differentiation and size. Osteoclast resorption is severely impaired in the D2J mice indicating that Osteoactivin plays a role in stimulating osteoclast resorption. Although controversy in the role of Osteoactivin in osteoclast differentiation remains, it seems that numerous sources confirm that Osteoactivin positively regulates osteoclast resorption. Further evidence into the mechanisms that regulate Osteoactivin mediated osteoclast differentiation need to be explored.

As previously described, Osteoactivin has shown to play a role in bone formation. Angiogenesis has been shown to be a critical factor in bone formation and fracture healing [357, 358]. Early evidence indicating the role of Osteoactivin in the endothelium arose from a study showing that dendritic cells expressing Osteoactivin bind to heparin sulfate proteoglycans on the surface of endothelial cells in an RGD dependent fashion [359]. Addition of recombinant Osteoactivin was shown to stimulate angiogenesis *in vitro* using human umbilical vein endothelial cells [360]. These studies reveal that Osteoactivin may play a role in angiogenesis. There exists a link between angiogenic factors and tumor growth. Osteoactivin extracellular domain has been shown to be shed from the membrane of breast cancer cells by ADAM10, a metalloproteinase involved in the shedding of several different molecules from the cell membrane, and stimulate the migration of endothelial cells [17]. Furthermore, this group also showed that Osteoactivin cooperates with neuropilin-1 and $\alpha_5\beta_1$ to potentiate VEGF signaling [327]. This indicates that Osteoactivin may play a role in regulating critical angiogenic factors such as VEGF. As previously described Osteoactivin has shown to play a role in tissue repair.

Osteoactivin has been shown to be highly expressed in a hyperoxia model and addition of recombinant Osteoactivin promoted endothelial tube formation *in vitro* and *in vivo* [361]. Osteoactivin has been shown to play a role in both angiogenesis and injury repair. It will be interesting to determine the role of Osteoactivin in the molecular mechanisms and cross-talk that exist between angiogenesis and tissue repair.

Several different cell types and growth factors are involved in the repair of a bone fracture. Osteoactivin has shown to play an important role in osteoblasts, osteoclasts, and angiogenesis. Osteoblasts, osteoclasts, and endothelial cells are all involved in the process of fracture repair; therefore, it would make sense that Osteoactivin would play a role in this process. Our group has shown that Osteoactivin is highly expressed in post fracture calluses in a fracture model [362]. In this study, Osteoactivin was expressed in osteoblasts, osteoclasts, and osteocytes at the bony callus indicating that Osteoactivin may play a role in all three cell types during repair. Furthermore, Osteoactivin was shown to stimulate bone regeneration in a critical size defect in the rat [363] and mouse [360] model.

Large fractures such as non-unions are difficult to treat and mostly require invasive surgeries. Several treatment options combining the use of scaffolds, stem cells, and growth factors are being developed to treat large bony defects [364]. Osteoactivin was shown to have comparable effects on MSC differentiation on cells grown on hydrogels asBMP-2 [365]. This study also showed that bolus rather than continuous administration produces a more profound result. Furthermore, MSCs taken from human placenta and fetal membrane show an enhancement in

osteogenic differentiation when stimulated with recombinant Osteoactivin [366]. This data indicates that Osteoactivin may be used as a potential treatment for the acceleration of fracture healing and can be combined with other biomaterials, cells, and growth factors as a delivery method.

Although there is an abundant amount of literature describing the role of Osteoactivin in bone remodeling, little is known about the Osteoactivin induced signaling mechanism behind osteoblast and osteoclast differentiation and function. Our group has shown that Osteoactivin interacts with $\alpha_v\beta_1$ and heparin sulfate proteoglycans (HSPG) to activate osteoblast adhesion [367]. Furthermore, another group has described that Osteoactivin interacts with FGF1R to stimulate downstream signaling [360]. In osteoclasts, only the signaling pathway involved in osteoclast resorption has been described. Previous literature has shown that Osteoactivin binds to $\alpha_v\beta_3$ and that addition of chondroitin sulfate-3 halts osteoclast resorption [10]. A novel receptor involved in both osteoblast and osteoclast differentiation has not yet been described. Downstream signaling molecules and regulatory genes involved in Osteoactivin mediated osteoblast and osteoclast differentiation have also not been described.

Table 2.3 Osteoactivin/GPNMB Animal Models

Protein/Gene	Animal Model	Phenotype	Reference
Osteoactivin-Tg	Transgenic-CMV Promoter	Increased trabecular bone volume due to enhanced osteoblast proliferation and differentiation. Protects skeletal muscle in cases of denervation	8,315
Osteoactivin-Tg^{TRAPC}	Transgenic-TRAPC Promoter	Decreased bone volume due to an enhancement in osteoclast differentiation and resorption	12
D2J	Mutant	Decreased trabecular bone volume at 8 and 16 weeks and increased cortical thickness; impaired osteoblast differentiation and osteoclast resorption but enhanced osteoclast differentiation	9,10
Osteoactivin-KO	Knockout	Increased bone mass at 4 and 12 weeks; impaired osteoblast differentiation and osteoclast function	Unpublished Data
SOD1^{G93}/GPNMB	Transgenic	Neuroprotective effect in neurodegenerative disease amytrophic lateral sclerosis (ALS)	319

2.9 CD44

Clustering domain-44 (CD44) is a type I transmembrane glycoprotein that participates in several cellular processes, including adhesion, survival, proliferation, differentiation, and migration [368]. It was first discovered in white blood cells and ranges in size from 80-200 kDa based on alternative splicing on its N-terminal extracellular domain. The standard CD44 isoform (CD44s) is the smallest and most basic isoform consisting of a heavily glycosylated amino terminal domain, a short stem structure, a transmembrane domain, and a short C-terminal domain with multiple motifs [368]. The amino terminal domain is a stretch of roughly 90 amino acids that is heavily glycosylated with several disulphide linkages forming a globular like structure. This portion of CD44 serves as the primary docking site for several extracellular matrix proteins, including hyaluronan, collagens, laminins, and fibronectins [369]. There have been other ligands that interact with CD44 including Osteopontin [370], Serglycin [371], and galectins [372]. The stem structure is a short 46 amino acid structure located adjacent to the plasma membrane. The stem size can vary based on the variant isoform (v1-10) and contains a few glycosylation sites important for heparin sulfate interactions [373]. The transmembrane domain of CD44 consists of 23 hydrophobic amino acids and is believed to be important for its incorporation into lipid raft complexes [374, 375]. The C-terminal domain is a short amino acid sequence; however, it contains multiple motifs that allow interaction with the actin cytoskeleton. Several actin cross-linking proteins from the Ezrin, Radixin, and Moesin (ERM) family and ankyrin have been shown to interact with the cytoplasmic tail of CD44 and link it with the cytoskeleton [376-378]. These interactions have been implicated to be involved in cell migration and adhesion.

The CD44 cytoplasmic tail also contains phosphorylation sites at Ser291 and Ser325 regulated by PKC, which influence its interactions with ERM proteins [379].

CD44 plays a role in several signal transduction pathways. Several pathological conditions, primarily cancer, have been associated with changes in CD44 and its signaling. There are three proposed mechanisms in which CD44 can stimulate signal transduction pathways. These mechanisms include acting as a ligand binding receptor by interacting with ECM proteins by serving as a docking platform, acting as a co-receptor to modulate the activation of other receptors, and lastly as an organizer of the cytoskeleton [368].

The stimulation of signal transduction pathways is a complex process. Upon stimulation, the receptors can be found to associate with and may require several other molecules or co-receptors in order to function. Co-receptor signaling involves binding of soluble ligands and regulation of ligand binding through corresponding receptors [380]. The function of CD44 as a co-receptor may provide the best evidence of how CD44 influences cellular signaling as CD44 does not have any intrinsic catalytic domains. The direct signal transfer, however, cannot be ruled out as CD44 has been shown to interact with several intracellular signaling proteins, such as Ras, Rho, Src, Fyn, and PKC, at its cytoplasmic tail [381-383]. CD44 has shown to interact with ERBB family members and mediate downstream signaling which is important for cell proliferation, survival, and differentiation [384]. Interestingly, CD44 has been shown to interact with several signal transduction pathways important for bone cell differentiation and function.

The first evidence of CD44 expression in bone was shown in osteocytes and was proposed as a marker for osteocyte formation [385]. Several other publications have shown that the CD44s protein is expressed in osteoblasts [386-388], mesenchymal stem cells [389], and osteoclasts [390]. Studies from CD44 deficient mice have shown an impairment in osteoblast differentiation *ex vivo* but no difference in osteoclast differentiation both *in vivo* or *in vitro* [391-393]. This data indicates that CD44 may be an important regulator of osteoblastogenesis. The level of CD44 in osteoblasts has shown to increase during differentiation [394]. This indicates that CD44 may play a role during osteoblast differentiation.

CD44 has shown to play a large role as a co-receptor in several signal transduction pathways important for osteoblast differentiation. CD44 has been shown to be involved in BMP signaling. Galectin-9 has been shown to induce osteoblast differentiation through the CD44/Smad pathway [395]. This group has shown that CD44 and BMPR clustering is necessary for Galectin-9 induced osteoblast differentiation through the Smad 1,5,8 pathway. Another group has stated that hyaluronic acid enhancement of osteoblast differentiation through CD44 downregulates BMP antagonists Noggin and Follistatin [396]. CD44 has also shown to be involved in the mechanisms connecting both osteoblasts and osteoclasts. Stimulation of CD44 by hyaluronan, the major ligand of CD44, enhanced the expression of Intracellular Adhesion Molecule-1 (ICAM-1) and Vascular Cell Adhesion Molecule-1 (VCAM-1) and that anti-CD44 antibody addition augmented its expression [397]. Furthermore, stimulation of CD44 in osteoblasts resulted in the adhesion of osteoblasts to monocytic

precursors indicating that CD44 may play a role in the intracellular signaling pathway that interlinks osteoblasts and osteoclasts.

CD44 has shown to play a large role in osteoclasts and osteoclast signaling. Several studies involving CD44 and osteoclasts have shown that CD44 interacts with $\alpha_v\beta_3$ and Osteopontin [398, 399]. Osteopontin has shown to stimulate osteoclast migration through CD44 and $\alpha_v\beta_3$ [400]. Furthermore, Osteopontin deficient mice are hypomotile and have decreased levels of CD44 indicating the importance of these two molecules in osteoclast migration [401]. There have been discrepancies involving the activation of CD44 and its role in osteoclast differentiation and function [402-404]. Previous literature has shown that CD44 stimulation by hyaluronan inhibits the migration of osteoclasts by down regulating MMPs [405]. This data indicates that CD44 activation may act as a “stop” signal for osteoclast resorption. Furthermore, it has been shown that CD44 ligands hyaluronan, chondroitin sulfate, and Osteopontin prevent macrophage/monocyte multinucleation [406]. This data indicates that CD44 activation may prevent fusion of multinucleated cells of the macrophage lineage. Overall the discrepancies in the activation of CD44 in osteoclasts can best be explained by ligand specific interactions.

Since CD44 has been shown to be involved in several different cell types in the bone microenvironment, activation of this receptor may play a role in several different bone pathologies. CD44 expression along with Osteopontin was shown to be highly expressed in osteoprogenitor cells, osteoclasts, and osteocytes in fracture calluses [407]. This indicates that CD44 may play a potential role in fracture healing. Migration of MSCs into the fracture site is critical in order for proper formation of new bone. As

discussed previously, Osteopontin and CD44 interact to stimulate the migration of a variety of cell types [398, 408, 409]. Osteopontin deficient mice have impaired fracture healing thought to be the result of reduced CD44 expression or the interaction with CD44 and other co-receptor interactions [410]. This data indicates that Osteopontin and CD44 interaction may be necessary for proper callus formation. During fracture repair or bone remodeling, osteocytes secrete chemotactic factors to stimulate the recruitment of stem cells. In a previous report, osteocytes under stress of hypoxia were shown to stimulate MSCs through an Osteopontin/CD44 mediated pathway [411]. Currently, therapies are being developed to target progenitor cells into areas of injury. A previous report has shown that the HA/CD44 interaction along with SDF-1 is important for the migration and homing of progenitor cells to the bone marrow [412]. Furthermore, *ex vivo* engineering of CD44 in human MSCs can target the cells to bone tissue [413]. It will be interesting to determine if targeting CD44 in MSCs and delivering them in large fracture defects will accelerate the healing. Future studies need to focus on the role of CD44 with other ligands in bone repair.

2.10 Lipid Raft

The proposed model of the phospholipid bilayer of the cell membrane is that all proteins are uniformly dispersed among a “sea” of lipids. This model predicts the plasma membrane as an unordered state; however, recent evidence suggests there exists regions within the membrane that are ordered that contain a specific and unique arrangement. Certain microdomains of the plasma membrane are enriched with sphingolipids and cholesterol allowing for less fluidity and a more ordered state. These microdomain structures are now termed lipid rafts, and their purpose is to organize the

plasma membrane into discrete microdomains for a variety of cellular functions [414]. Lipid rafts can serve a variety of functions, including vesicular trafficking endocytosis, regulation of cholesterol and calcium homeostasis, and stimulating signal transduction pathways. Caveolin-1 was the first protein to be discovered within the lipid raft, and integration with these proteins results in the formation of caveolae [415]. One of the key characteristics of lipid rafts is that it contains several proteins important for intracellular signaling. The proteins found within the lipid raft must have a particular affinity to exist within them. Examples of these proteins include GPI-anchored proteins, cholesterol-linked proteins, double acylated proteins (Src family kinases), and palmitoylated transmembrane proteins [416, 417]. Many of these proteins act as receptors in several signal transduction pathways related to bone. Recently there has been a new understanding in the role of the lipid raft in bone.

Caveolin proteins are a major component in the formation of lipid raft microdomains. There are three distinct caveolin proteins (Caveolin-1, Caveolin-2, Caveolin-3) that have been identified within the lipid raft [418]. The role of these proteins in bone is just recently being understood. Previous literature has shown that murine and human osteoblasts express high levels of caveolin proteins, predominantly Caveolin-1 and Caveolin-2 [419, 420]. The caveolin rich lipid rafts were also shown to have several important signal transduction molecules, including PDGF receptor, Src family of nonreceptor tyrosine kinase, Fyn,Ras, and other heterotrimeric G proteins [421]. This indicates that lipid rafts may be important for signal transduction pathways related to osteoblast differentiation and function.

Different groups have shown controversial results when determining the role of Caveolin-1 in osteoblasts. Caveolin-1 deficient mice were shown to have increased bone size and stiffness compared to controls [86]. These mice had significantly higher bone mass by microCT at 5 and 8 weeks of age and had impaired osteoblastogenesis with no effect on osteoclasts *ex vivo*. This indicates that Caveolin-1 may play a role in negatively regulating osteoblast differentiation. Another group has shown that Caveolin-1 is highly expressed in extracellular matrix vesicles, and that Caveolin-1 expression increases over the course of osteoblast differentiation [422]. Furthermore, overexpression of Caveolin-1 in MC3T3-E1 cells resulted in enhanced mineralization while siRNA treatment inhibited mineralization in a Src dependent manner. This data indicates that Caveolin-1 plays a large role in late stage osteoblast differentiation. Previous reports have shown that calcium signaling is important for osteoblasts and that there are receptors along the plasma membrane responsible for determining calcium levels in the extracellular environment [423, 424]. Calcium sensing-receptors (CSR) have been shown to be regulated by and complex with Caveolin-1 in human osteosarcoma cells [425]. This indicates that caveolins may play a role in regulating osteoblasts through their interaction with calcium receptors.

Caveolin proteins have also shown to play a role in osteoclasts. Previous literature has shown that both Caveolin-1 and Caveolin-2 are highly expressed upon RANKL stimulation in osteoclasts and that disruption of lipid raft integrity led to reduced osteoclast formation [426]. This notion is supported by the idea that disruption of the lipid raft inhibits the RANK signaling axis and that bone resorption and actin ring formation are highly dependent on lipid raft membrane activity [427]. Furthermore,

osteoclast formation and survival are highly reliant on a source of exogenous cholesterol, as depletion of cholesterol in the lipid raft membrane by the drug methyl-β-cyclodextrin (MβCD) results in increased osteoclast apoptosis [428]. These data indicate that the lipid raft and its integrity play numerous roles in osteoclasts that include differentiation, resorption, and survival.

The lipid raft has been shown to play a large role in signal transduction pathways. Several of these pathways have been shown to be related to bone cell differentiation and function. The activation of receptors can result in the recruitment of these activated receptors along with other signaling molecules within the lipid raft; for example, a previous report has shown that activation of FGFR2 results in the recruitment of FGFR2 and Cbl into lipid rafts [429]. Upon FGFR2 activation, Cbl binds to PI3K and attenuates its response, resulting in decreased osteoblast survival. This indicates that the lipid raft may play a role in the regulation of osteoblast survival. BMP signaling has also been shown to play a role in lipid raft signaling in osteoblasts. Treatment of MC3T3-E1 cells with recombinant BMP-2 resulted in cell migration, which was abolished upon MβCD treatment and restored upon exogenous cholesterol addition [430]. Furthermore, BMP-2 addition increased the incorporation of β_1 integrin into lipid rafts, indicating that the lipid raft may be important for cell adhesion and migration. Lipid raft signaling has also shown to be important for osteoblast proliferation [431]. In this study, the addition of Galectin-9, a β-galactoside-binding lectin, led to an increase in osteoblast proliferation in a Src/ERK dependent mechanism. Furthermore, addition of Galectin-9 induced clustering of lipid rafts and disruption of lipid rafts by MβCD treatment led to an inhibition of Galectin-9 mediated osteoblast proliferation. These

results indicate that certain growth factors can stimulate a variety of cellular functions, including proliferation, migration, adhesion, and viability through lipid raft signaling.

The role of the lipid raft and the family of caveolin proteins in bone cells has only recently been discovered. Although caveolin proteins and lipid rafts have been studied for some time now, their role in musculoskeletal physiology and disease state is now only recently starting to be understood. Most of the literature that has been described on the role of caveolins in disease states has come from muscle. Caveolin-3, the predominant caveolin in muscle, has been linked to several diseases [418]. Mutations within Caveolin-3 have been linked with the muscle diseases autosomal dominant Limb Girdle Muscular Dystrophy (LGMD), rippling muscle disease, hyper-CKemia, and distal myopathy [432-434]. Interestingly, caveolins, in particular Caveolin-1, are highly expressed during musculoskeletal repair. In muscle, overexpression of Caveolin-1 in satellite cells -the cells that are activated in response to injury- fail to differentiate into multinucleated myotubes [435]. In bone, Caveolin-1 expression was found to be upregulated in peripheral blood mononuclear cells (PBMNs) during fracture repair [436]. These results show that Caveolin-1 may have a potential role as a therapeutic for musculoskeletal repair.

Chapter 3

Materials and Methods

3.1 Cell Culture and Reagents

The MC3T3-E1 osteoblast like cell line was purchased from ATCC (Manassas, VA). Recombinant Osteoactivin was purchased from R&D Technologies (Minneapolis, MN). The osteogenic differentiation factors β-Glycerophosphate, L-Ascorbic Acid, and Dexamethasone were purchased from Sigma (St. Louis, MO). The Osteoactivin antibodies used were purchased from Bioss (Woburn, MA) and R&D Technologies. The CD44 antibody was purchased from Calbiochem (San Diego, CA). The antibodies pERK, ERK, pP38, P38, GAPDH were purchased from Cell Signaling (Danvers, MA). The β-Actin, Ki-67, and Tubulin antibodies were purchased from Bioss. Caveolin-1 antibody was from Assay Biotechnology (Sunnyvale, CA). Secondary fluorescent antibodies Alex flour-488 and Alexa flour-594 were purchased from Cell Signaling. The ERK inhibitor U0126 was purchased from Cell Signaling. Lipid raft inhibitors nystatin and Methyl-β-cyclodextrin (MβCD) were purchased from Calbiochem and Sigma respectively. Alexa flour-488-labeled cholera toxin B, deoxycholic acid, and Percoll® were purchased from Sigma. The trichloroacetic acid and tricene were purchased from Fisher Scientific (Waltham, MA). DAPI used for immunofluorescence was purchased from Abcam (Cambridge, UK). The rhodamine phalloidin was purchased from Cytoskeleton, Inc. (Denver, CO). Tween-20 was purchased from Fisher Scientific.

RANKL, M-CSF, and Hyaluronan (High Molecular Weight) were purchased from R&D Technologies. The antibodies pPLC$_\gamma$2, PLC$_\gamma$2, pI$_\kappa$β, I$_\kappa$β, pJNK, JNK, pERK, ERK, pP38, P38, pAKT, AKT, NFAT, LC3,and GAPDH were purchased from Cell Signaling (Danvers, MA). The *p*-nitrophenyl phosphate (*p*-NPP), Fast Red Violet Salt, and WGA Lectin HRP (from *Triticum vulgaris*) were all purchased from Sigma (St. Louis, MO).

3.2 Mice

C57Blk6 (WT) and CD44$^{-/-}$ (CD44KO-global) were purchased from Jackson Laboratory. Both 8 and 16 week males were used. All mouse colonies were housed and maintained at Northeast Ohio Medical University according to the guidelines set by the Institutional Animal Care and Use Committee (IACUC).

3.3 MicroCT

Femurs from 8 and 16-week-old male WT and CD44KO mice (*n*=6) were analyzed using a SkyScan 1172 high-resolution microtomography system (Bruker, Billerica, MA). Trabecular measurements of femurs were analyzed as previously described [6, 437]. Briefly, trabecular measurements were taken 400 µm below the distal growth plate in 750 consecutive slices of 7.6-µm resolution over a distance of 5,700 µm. The volumetric regions were rendered as 3D arrays using SkyScan NRecon software. Percentage of bone volume per tissue volume (BV/TV; %), bone area (B.Ar; mm^2), bone perimeter (B.Pm; mm) trabecular number (Tb.N; no./mm), trabecular separation (Tb.Sp.; mm), and trabecular thickness (Tb.Th; mm) were measured and calculated using SkyScan CT software. Three-dimensional reconstructed images of the

saggital and axial planes of the femoral metaphysis were generated using SkyScan CTvox software version 2.4.

3.4 ELISA

Serum samples were obtained from 8- and 16-week-old male WT and CD44KO mice ($n \geq 5$). Serum P1NP (MyBiosource, San Diego, CA), Osteoclacin (Biomedical Technologies, Stoughton, MA), CTX-1 (MyBiosource), RANKL, and OPG (R&D Technologies) were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's guidelines. The Osteoactivin ELISA (R&D) was also used to determine the concentration of the protein in osteoclast cell cultures.

3.5 Tissue Preparation and Bone Histomorphometry

Femurs from 8-week-old male WT and CD44KO (n≥4) were dissected, fixed in 4% paraformaldehyde, dehydrated, and embedded undecalcified in plastic methylmethacrylate resin as previously described [6]. Saggital sections (5 µm thick) were stained with Von Kossa-toluidine blue [438]. Quantitative histomorphometry was performed in an area from 100-600 µm proximal to the growth plate using OsteoMeasure software version 3.2.1 (Osteometrics, Decatur, GA) as previously described [439]. Images were acquired with a brightfield microscope (Olympus, Center Valley, PA). Three-dimensional and two-dimensional parameters were measured and calculated in three sections per animal. The 3D parameters were trabecular number (no./mm) [Tb.N=(4π) x 0.5 x (B.Pm/T.Ar)] and trabecular separation (µm) [Tb.Sp= (1/Tb.N) x (1,000-Tb.Th)]. The 2D parameters were bone perimeter per tissue area (B.Pm/T.Ar; mm/mm^2), trabecular number per tissue area (Tb.N/T.Ar; no./mm^2),

osteoblast number per bone perimeter (N.Ob/B.Pm no./mm), and percent osteoblast surface per bone surface (Ob.S/B.S.;%).

For dynamic histomorphometry, 7-week-old male WT and CD44KO mice (*n*=4 per group) were injected intraperitoneally with 10 mg/kg calcein AM (ThermoFisher Scientific) 7 and 2 days before termination. Femurs were collected and histomorphometric analysis of undecalcified sections of the distal femur using a Nikon Eclipse Ti epifluorescent microscope (Nikon, Melville, NY; Tokyo, Japan) was performed. Single-labeled surface, double-labeled surface, mineralizing surface [MS=dLS + (sLS/2)], mineral apposition rate (MAR) and bone formation rate (BFR; MAR X MS) were measured and calculated.

3.6 Osteoclast Culture

Mouse bone marrow-derived stem cells were isolated from 8 week male WT and CD44KO mice as previously describe [7]. To generate osteoclasts, bone marrow cells were plated at a cell density of $1.2X10^5$ cells in a 96 well plate and primed with M-CSF (20ng/mL) for the first 3 days. M-CSF dependant bone marrow macrophages (BMM) or osteoclast precursors (OCP) were then given RANKL (40ng/mL) and M-CSF (20ng/mL) and a second dose of RANKL and M-CSF was given again 48 hours later. In parallel cultures, OCP were treated with recombinant Osteoactivin along with RANKL and M-CSF. At day 7 cells were fixed, and TRAP activity and staining was assessed. TRAP positive osteoclast (n>3 nuclei) images were taken using a Nikon Ti Eclipse inverted microscope. TRAP positive osteoclast differential count and size were calculated using the NIS-Elements software.

3.7 TRAP Staining and Activity

Mature osteoclasts plated in 96 well plates were fixed with 10% formalin, washed with dH_20, and allowed to air dry. For TRAP activity assays Methanol:Acetone was added to cultures followed by incubation with TRAP buffer (52 mM of Na-tartrate in 0.1 M Na-acetate buffer pH 5.2) containing 0.1 mg/mL of *p*-nitrophenyl phosphate (*p*-NPP). The reaction was stopped by adding 1 N NaOH and read at an potical density of 405 nm using a BioTek Synergy microplate reader. For TRAP staining, mature osteoclasts were incubated with TRAP buffer containing 1.5 mM naphthol AX-MX phosphate and 0.5 mM Fast Red Violet LB Salt.

3.8 In vitro Osteoclast Resorption Assays

BMM isolated from WT and CD44KO were plated on Corning ® OsteoAssay surfaces (Corning) for 4 days with M-CSF (20ng/mL) and RANKL (50ng/mL). In parallel cultures, rOA (100ng/mL) was added to cultures 48 hours before termination. On the fourth day, osteoclast cultures were terminated using 10% bleach. Resorption from osteoclasts was analyzed by quantitation of the resorbed area of the well over the total area using the NIS-Elements software. Additionally, BMM from WT and CD44KO were cultured on collagen coated 6 well plates and given M-CSF (20ng/mL) and RANKL (50ng/mL) for 48 hours. Mature osteoclasts on collagen coated plates were removed using 2.5mg/mL collagenase in dissociation buffer (Life technologies) and seeded on bovine cortical slices in 96 well plates along with M-CSF and RANKL. In parallel cultures, rOA (100ng/mL) was added to osteoclasts seeded on cortical slices. Cells were fixed with 4% paraformaldehyde, permeabilized with triton X-100, and incubated

with rhodamine phalloidin followed by counterstaining with DAPI mounting media to visualize actin ring formation. Images were taken using the Olympus 100 confocal microscope. After staining for actin ring formation, osteoclasts on cortical slices were TRAP stained and counted (n>3 nuclei) for each condition. For analysis of the resorption pits on the cortical slices, osteoclasts were removed with a soft brush and slices were incubated with 200µg/mL lectin-HRP for one hour at room temperature. Slices were then incubated with 0.52mg/mL 3,3'-diaminobenzidine for 30 minutes. Images were taken using a Nikon Ti Eclipse inverted microscope and resorption pit area and %resorbed area were measured sing the NIS-elements software.

3.9 Calvarial Bone Resorption Assay

A mouse model of RANKL-induced bone loss from the calvaria was used as previously described [440]. A collagen sheet (100mm^2) (Medline) was soaked with PBS (control), RANKL (50µg/mL), or RANKL and rOA (100µg/mL) in 30µL volume and placed on the center of the calvaria in 5 week WT or CD44KO mice (n=5). After 7 days, the mice were sacrificed and the calvaria were removed and fixed in 4% paraformaldehyde and stained for TRAP. Images were taken using a Nikon SMZ 800 stereomicroscope. The TRAP + stained area was quantified using the NIS-Elements analysis software. Micro-computated tomography (µCT) was also performed using the SkyScan 1172 microCT system (Skyscan, Aartselaar, Belgium; 72kV, 113µA, 7.7-µm sections). Three dimensional reconstructed images of the calvaria were generated using SkyScan CTvox software.

3.10 Immunofluorescence

WT primary osteoblasts, MC3T3-E1 cells, or BMM form WT mice were cultured in 4-well 1.7 cm^2 chamber slides (BD Falcon, Corning, NY) with α-MEM media supplemented with 10% serum. Cells were then fixed with 4% paraformaldehyde (PFA) and permeabilized using 0.5% Triton X-100 in PBST (Tween-20; 0.1%). Primary osteoblasts were then blocked with 5% BSA in PBST followed by overnight incubation of the primary antibodies CD44 (1:500), Osteoactivin (1:250), Caveolin-1 (1:250). The next day corresponding secondary antibodies Alexa flour-488 and Alexa flour-568 (Cell Signaling) were incubated for 2 hours in a humidified chamber followed by counterstaining with diamidino-2-phenylindole (DAPI) mounting media. For visualization of the lipid raft Alexa flour-488 conjugated CTX-B (1:100) was added for 2 hours at room temperature prior to termination. Images were taken using a Nikon Ti Eclipse inverted microscope.

3.11 Mass Spectroscopy Analysis

Biotinylation of recombinant Osteoactivin (R&D Technologies) was carried out using the EZ-Link NHS-Biotin Kit (ThermoScientific). Briefly, 25 µg/mL recombinant Osteoactivin was added to 10 mM Biotin dissolved in DMSO and allowed to incubate for 2 hours at room temperature and dialyzed overnight. Freshly labeled biotinylated Osteoactivin was then added to protein cell lysates in RIPA buffer and allowed to incubate for 1 hour at 4^0C on an orbital rocker. To purify biotinylated proteins, the NHS-Biotin labeled cell lysates were incubated with streptavidin beads (Pierce) for 1 hour at 4^0C on an orbital rocker. The cell lysate slurry was then transferred to an Ultrafree-MC

centrifugal filter tube and subjected to a series of 3 washes using RIPA lysis buffer. The purified labeled protein was then eluted with β-mercaptoethanol in laemelli buffer boiled at 95^0C and proteins separated by SDS-PAGE. The gel was then silver stained, digested and analyzed by LC-MS/MS Mass Spectroscopy by the Ohio State Spectrometry and Proteomics Facility. The tandem mass spectrometry data were processed using Mascot Distiller to form a peaklist and analyzed using the MASCOT tandem mass spectrometry search engine and Turbo SEQUEST algorithm in the BioWorks 3.1 software as previously described [441].

3.12 Immunoprecipitation

Total cell lysate from primary osteoblast and pre-osteoclast culture was pre-cleared with 20 μl protein A-Sepharose beads (Sigma; 50% slurry in PBS) end-over-end at 4ºC for 1 hour. Cell lysates were then incubated with 3 μg anti-CD44 antibody or corresponding IgG isotype control (Abcam) end-over-end at 4ºC overnight. The immune-complexes were captured by 25 μl protein A-Sepharose beads with end-over-end mixing for 1 hour at 4ºC. Beads were washed four times by centrifugation (400x g, 5 minute) in cell lysis buffer and then boiled at 95ºC for 5 minutes in 20 μl reducing SDS-sample buffer inducing the elution of the captured proteins. Beads were removed by centrifugation, and the supernatant was analyzed by Western blot.

3.13 Western Blot Analysis

Total protein was isolated from osteoblast cultures using radioimmunoprecipitation (RIPA) assay buffer (Millipore, Billerica, MA) with phosphatase inhibitor cocktail (Pierce, Rockford, IL). Protein concentration was

determined using the Pierce BCA protein Assay kit. Protein samples (25-40µg) were subjected to separation SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (BioRad, Hercules, CA) by the Trans-Blot Turbo system (BioRad). Membranes were probed overnight with the following primary and secondary antibodies as listed in Table 3.1 and Table respectively. The signal was developed with chemiluminescent substrate (Pierce) and detected using the Syngene PXi system (Syngene, Rockville, MD). Densitometric analysis was performed by the optical density of the phosphorylated protein over the total using the Syngene software analysis.

Table 3.1 List of Primary Antibodies

Primary Antibody	**Source**	**Company**
Osteoactivin	Rabbit	Bioss, R&D Technologies
CD44	Rat	Calbiochem
pERK	Rabbit	Cell Signaling
ERK	Rabbit	Cell Signaling
pP38	Mouse	Cell Signaling
P38	Mouse	Cell Signaling
pJNK	Rabbit	Cell Signaling
JNK	Rabbit	Cell Signaling
pIκβ	Rabbit	Cell Signaling
Iκβ	Rabbit	Cell Signaling
pPLCγ2	Rabbit	Cell Signaling
PLCγ2	Rabbit	Cell Signaling
pAKT	Rabbit	Cell Signaling
AKT	Mouse	Cell Signaling
NFAT2	Rabbit	Cell Signaling
LC3	Rabbit	Cell Signaling
GAPDH	Rabbit	Cell Signaling
Tubulin	Rabbit	Bioss
β-Actin	Rabbit	Bioss
Ki-67	Rabbit	Bioss

Table 3.2 List of Secondary Antibodies

Secondary Antibody	Conjugate	Company
Anti-Rabbit	HRP	Cell Signaling
Anti-Rat	HRP	Cell Signaling
Anti-Mouse	HRP	Cell Signaling
Anti-Rabbit	Alexa flour-488	Cell Signaling
Anti-Rat	Alexa flour-594	Cell Signaling

3.14 Transfection

MC3T3-E1 cells were transfected with 100 nM scramble siRNA or CD44 siRNA (Dharmacon, Lafayette, CO) using Lipofectamine (Invitrogen). After 48 hours, cells were treated with recombinant Osteoactivin, ERK inhibitors (10μM), or in combination for 24 hours. After the 24 hour period RNA was isolated and qPCR was performed. For Western blot analysis, cells were treated with recombinant Osteoactivin for 10 minutes and protein isolated by RIPA. The efficiency of CD44 knockdown was determined by Western blot analysis.

3.15 Quantification of Matrix Deposition and Mineralization

Matrix deposition and maturation in osteoblast and bone marrow derived mesenchymal stem cell cultures were determined by ALP staining and activity. ALP staining was performed at day 7 using an ALP staining kit (Sigma), according to the manufacturer's protocol. ALP-positive cells are stained purple. In parallel cultures, ALP activity was determined using an ALP activity kit (Anaspec, Fremont, CA) based on the manufacturer's recommendations. Briefly, 100 μL of total protein cell lysate from each sample was added to a 96 well plate in triplicates along with 50 μL working solution.

Absorbances were measured over time starting at 0 to 60 minutes. ALP activity is expressed nmol per minute per total microgram protein. Absorbance values were normalized to the total protein using BCA.

In order to assess mineralization, osteoblasts were differentiated in culture for 21 days and stained for Von kossa. Briefly, cells were fixed with 10% formalin for 20 minutes and stained with 5% silver nitrate solution under UV exposure for 30 minutes. Cells were washed 3 times followed by the addition of sodium carbonate and 5% sodium thiosulfate to visualize the mineralized nodules. Mineralized nodule count was assessed using the Nikon Ti Eclipse analysis software.

3.16 RT-qPCR

Total RNA was isolated from proliferating and differentiated osteoblasts grown in 6 well dishes by adding 1 mL Qiazol (QIAGEN, Hilden, Germany). Total RNA was purified using an RNA extraction kit (QIAGEN). RNA concentrations were determined using a NanoDrop 2000 Spectrophotometer (ThermoScientific). Following RNA isolation cDNA was prepared using a High Capacity cDNA Reverse Transcription kit (Life Technologies). Quantitative (q) RT-PCR was performed with the Step-one qPCR system in duplicate with 2X SYBR Green PCR Master Mix (Life Technologies). qPCR cycles consisted of an initial 50^{o}C cycle for 2 minutes, followed by a second cycle of 95^{o}C for 10 minutes, followed by 40 cycles of 95^{o}C for 15 seconds, and a final cycle of 60^{o}C for 1 minute. Samples were analyzed using a 7900 Fast Real Time PCR System (Applied Biosystems), and relative gene expression was determined using the ▲▲C_T method using GAPDH as an internal control. The sequences for oligonucleotide primers

(Integrated DNA technologies, Coralville, IA) for osteoblasts and osteoclasts are lasted in Table 3.2 and Table 3.3, respectively.

Table 3.3 Osteoblast Primers used for qPCR

Primers	**Sequence**
Runx2	5'-GACGAGGCAAGAGTTTCACC-3' (Runx2 forward) 5'-GGACCGTCCACTGTCACTTT-3' (Runx2 reverse)
Osterix	5'-GCAACTGGCTAGGTGGTG GTC-3' (Osterix forward) 5'-GCAAAGTCAGATGGGTAAGTAGGC-3' (Osterix reverse)
Alkaline Phosphatase (ALP)	5'-CCGATGGCACACCTGCTT-3' (ALP forward) 5'-GGAGGCATACGCCATCACAT-3' (ALP reverse)
Osteocalcin (OCN)	5'-CTGACAAAGCCTTCATGTCCAA-3' (Osteocalcin forward) 5'-GCGCCGGAGTCTGTTCACTA-3' (Osteocalcin reverse)
Type I Collagen (Col1α1)	5'-CCTGAGTCAGCAGATTGAGAACA-3' (Col1α1 forward) 5'-CCAGTACTCTCCGCTCTTCCA-3' (Col1α1 reverse)
Osteoactivin	5'-AATGGGTCTGGCACCTACTG-3' (Osteoactivin forward) 5'-GGCTTGTACGCCTTGTGTTT-3' (Osteoactivin reverse)

Table 3.4 Osteoclast Primers used for qPCR

Primers	**Sequence**
RANK	5'-AGTTTAAGCCAGTGCTTCACG-3' (RANK forward) 5'-ACGTAGACCACGATG ATGTCG-3' (RANK reverse)
TRAP	5'-GCAGTATCTTCAG GACGAGAAC-3' (TRAP forward) 5'-TCCATAGTGAAACCGCAAGTAG-3' (TRAP reverse)
DC-STAMP	5'-TGTATCGGCTCA TCTCCTCCAT-3' (DC-STAMP forward) 5'-GACTCCTTGGGTTCCTTGCTT-3' (DC-STAMP reverse)
NFATc1	5′-CTCGAAAGACAGCAC TGGAGCAT-3′ (NFATc1 forward) 5′-CGG CTGCCTTCCGTCTCATAG-3' (NFATc1 reverse)
OSCAR	5′-CTGCTGGTAACGGATCAGTC CCCAGA-3′ (OSCAR forward) 5′-CCAAGGAGCCAGAACCTTCGAAACT-3'(OSCAR reverse)
Calcitonin Receptor (Calcitonin Rec.)	5′-AGTTGCCCTCTTATGAAGGAGAAG-3′ (Calcitonin Receptor forward) 5′-GGAGTGTCGTCCCAGCACAT-3′ (Calcitonin Receptor reverse).

3.17 Lipid Raft Isolation

Membrane raft proteins were isolated by sucrose density fractionation as previously described [442]. Briefly, MC3T3-E1 cells were isolated and scraped into an ice-cold detergent-free Tricine Buffer and centrifuged to eliminate the nuclear material. The supernatant was removed and mixed with 30% Percoll® diluted in Tricene buffer and centrifuged at high speed (77,000 g) for 30 minutes. The separated plasma membranes were collected, sonicated, and mixed with 60% sucrose before being overlaid with a 35-5% step sucrose gradient and subjected to overnight centrifugation

(88,000g). Fractions were collected from the top sucrose layer, and proteins were precipitated using 0.1% deoxycholic acid in trichloroacetic acid. Samples were then ready to be used for SDS-PAGE and immunoblotting.

3.18 Luciferase Analysis

The Osterix (*Osx;* -1269/+91) luciferase construct was a kind gift donated by Mark Nanes (Emory University). Briefly, MC3T3-E1 cells were plated at $2.5x10^5$ cells in 6 well plates and transfected with either *Osx* or PGL3 basic (control) using Lipofectamine2000 (Invitrogen, Carlsbad, CA). Forty-eight hours after transfection, cells were harvested and assayed using firefly luciferase and *Renilla* luciferase substrates in the Dual Luciferase assay system (Promega, Madison, WI). The luciferase values were normalized based on *Renilla* values to correct for variation in transfection efficiency.

3.19 Cell Proliferation and Viability Assays

For proliferation assays, MC3T3-E1 cells were plated at $5.0 x10^3$ cells in 96 well plates in 6 replicates using α-MEM media supplemented with 10% serum. Cells were switched to low serum media (2.5% FBS) alone (control) or supplemented with different concentrations of recombinant Osteoactivin for 48 hours. Total DNA content was measured using a CyQuant NF cell proliferation assay kit (Life Technologies, Carlsbad, CA), according to the manufacturer's guidelines. Briefly, the media was aspirated and replaced with 1X dye binding solution and incubated at 37^oC for 1 hour. Flourescent intensity was measured using the BioTek Synergy H4 microplate reader (BioTek Instruments, Winooski, VT) with excitation at 485 nm and emission detection 530 nm. Furthermore, the number of Ki-67 % cells was quantified as previously described [443].

Briefly, MC3T3-E1 cells were plated at a density of $5.0x10^3$ on coverslips and cultured with α-MEM media supplemented with 10% serum. Cell were switched to low serum for 24 hours and then treated with different concentrations of recombinant Osteoactivin for an additional 24 hours. Cells were then fixed with 4% PFA, permeabilized with Triton X-100 in PBST, and blocked with 5% BSA in PBST for 1 hour. Cells were then incubated with anti-Ki-67 (Bioss) overnight. The next day cells were washed 3X, incubated with secondary Alexa flour-568 (Abcam), and counterstained with DAPI mounting media. The total number of Ki-67 + cells were counted and calculated as a percent over the total number of nuclei (Ki-67 % positive cells) at 10X magnification using a Nikon Eclipse Ti epifluorescent microscope. A minimum of 200 total cells were counted per condition in triplicates.

For cell viability assays, MC3T3-E1 cells were cultured in 96 well plates using α-MEM media supplemented with 10% serum. Cells were switched to low serum media (2.5% FBS) alone (control) or supplemented with different concentrations of recombinant Osteoactivin for 48 hours, and viability was assessed using Alamar blue as previously described [444]. Briefly, medium was aspirated and replaced with a 10% Alamar blue solution diluted in 1X Hank's media. Cells were incubated at 37^{o}C for 4 hours, and reduction of Alamar blue was determined colorimetrically by use of a spectrophotometer. The percent viability was expressed as fluorescence counts in the presence of recombinant Osteoactivin as a percentage of that in the control. For the cell apoptosis assay, levels of Caspase 3 in MC3T3-E1 cells were determined using the Caspase-Glo 3/7 assay (Promega). Briefly, MC3T3-E1 cells were cultured with α-MEM media supplemented with 10% serum and then switched to low serum media (2.5%

FBS) alone (control) or supplemented with 50 ng/mL recombinant Osteoactivin for 48 hours. Medium was removed and replaced with Caspase-Glo 3/7 reagent, and cells were then incubated at 37^{o}C for 4 hours. Luciferase activity was measured using a BioTek plate reader with excitation at 485 nm and emission 530.

3.20 Statistical Analysis

For all generated data, differences between individual groups were analyzed using the Prism 5 software version 5.04 (GraphPad, La Jolla, CA). Individual experiments were repeated a minimum of three times with similar results. In cases when multiple groups were being compared, a one-way analysis of variance (1-way ANOVA) was employed followed by a Tukey's multiple comparison post hoc test. In the cases involving the comparison of two groups, an unpaired t-test was performed. All differences where $p<0.05$ were regarded as statistically significant. Group means or means ± standard error of the mean (±SEM) was graphed.

Chapter 4

Results

4.1 Mesenchymal Stem Cell and Osteoblast Differentiation

Mesenchymal stem cells (MSCs) have the ability to differentiate into several different lineages important for musculoskeletal development and repair. In order for MSCs to be able to differentiate into a specific cell lineage, certain transcription factors must be expressed (Figure 4.1). For the osteoblast lineage, Runx2 and Osterix are the critical transcription factors important for MSC differentiation into osteoblasts. Once MSCs have committed to the osteoblast lineage, the osteoblasts undergo a specific series of differentiation stages.

During osteoblast differentiation several important growth factors are expressed that can serve a variety of roles important for osteoblast differentiation and function. Osteoactivin is a type I transmembrane glycoprotein shown to be expressed in both osteoblasts and osteoclasts. The role of Osteoactivin and its importance in osteoblast differentiation and function both *in vivo* and *in vitro* have already been described [3-6]; however, the signaling mechanism involved in Osteoactivin mediated osteoblast differentiation and function has not yet been investigated.

In this study we used the recombinant Osteoactivin (rOA) to study the role of Osteoactivin mediated signaling in both MSCs and osteoblasts. The recombinant Osteoactivin is the entire extracellular portion of the molecule and contains several domains and glycosylation sites. Our data shows that the recombinant Osteoactivin stimulates osteoblast differentiation through a CD44 dependent mechanism.

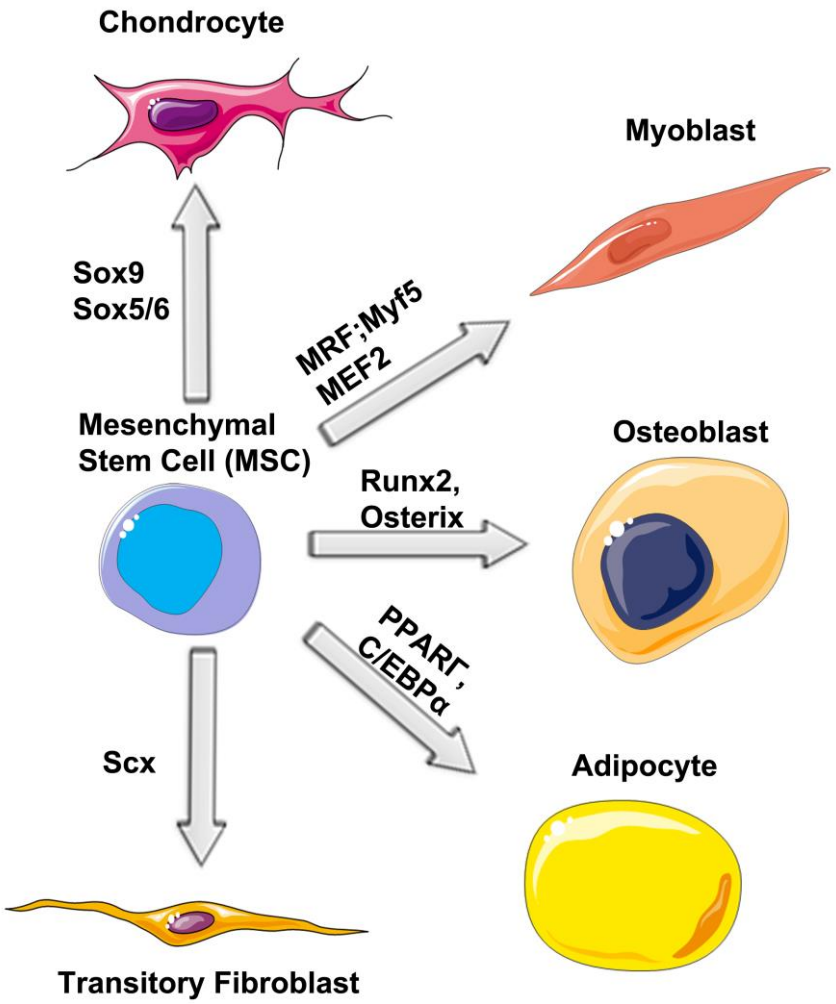


Figure 4.1: Schematic diagram showing the potential lineages of mesenchymal stem cell differentiation. There are a variety of different cell types important for musculoskeletal development. The above diagram depicts the different cell types that arise from the same MSC cell lineage and the critical transcription factors (next to arrows) necessary in order for those cells to differentiate into that particular cell type.

4.2 Osteoactivin has no Effect on MC3T3-E1 Osteoblast Proliferation and Viability

In order to investigate the role of recombinant Osteoactivin in osteoblast function, we wanted to determine its effect on osteoblast proliferation and viability using the MC3T3-E1 osteoblast-like cell line. We examined the role of recombinant Osteoactivin in osteoblast proliferation using CyQuant assay and Ki-67 immunoflourescent staining. CyQuant quantitatively measures the total DNA content, whereas Ki-67 staining indicates cells in a proliferating, non-quiescent state [445]. Both CyQuant assay (Figure 4.2A) and Ki-67 staining (Figure 4.2B) revealed that there were no significant changes in osteoblast proliferation with increasing concentrations of recombinant Osteoactivin. This indicates that recombinant Osteoactivin has no effect on osteoblast proliferation.

Next, we examined the role of recombinant Osteoactivin on osteoblast viability using the Alamar blue and Caspase 3/7 apoptosis Glo assays. The Alamar blue assay is an assessment of mitochondrial activity that fluoresces and changes color upon reduction in living cells, whereas the Caspase 3/7 Glo assay measures the activity of these enzymes which are important for cell apoptosis [444, 446]. Both Alamar blue (Figure 4.3A) and Caspase 3/7 Glo (Figure 4.3B) assays revealed no significant differences in cell viability when osteoblasts were treated with recombinant Osteoactivin. This indicates that recombinant Osteoactivin has no effect on osteoblast viability and survival.

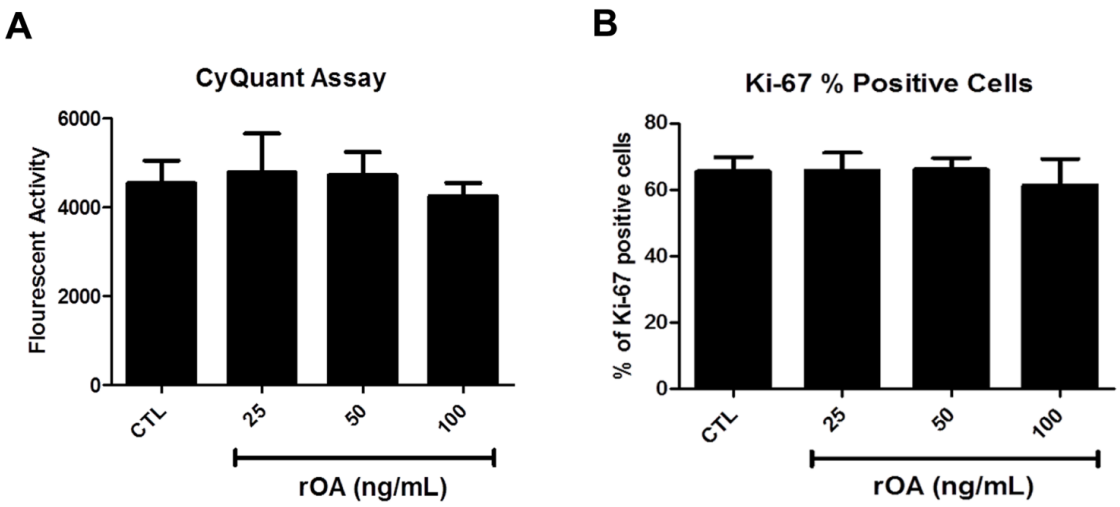


Figure 4.2: Recombinant Osteoactivin has no effect on MC3T3-E1 proliferation.

(**A**) CyQuant analysis of MC3T3-E1 cells plated at a seeding density of 5,000 cells per well in 96 well plates and serum starved overnight prior to addition of recombinant Osteoactivin for 48 hours. Following the 48 hour incubation period, proliferating cells were measured by quantitating the fluorescently-labeled DNA. (**B**) Quantitation of fluorescently labeled MC3T3-E1 cells with Ki-67. Cells were plated on coverslips and either left untreated (control;CTL) or treated with different concentrations of recombinant Osteoactivin for 24 hours. Cells were then fixed and incubated with a primary fluorescent antibody against the nuclear antigen Ki-67 followed by secondary incubation with Alexa-flour 594 and DAPI. The proportion of Ki-67 positive cells over the total number of nuclei (DAPI) was calculated. The experiments were run in triplicates and with 3 replicates per condition with similar results. Data presented in all graphs represent Mean ± SEM.

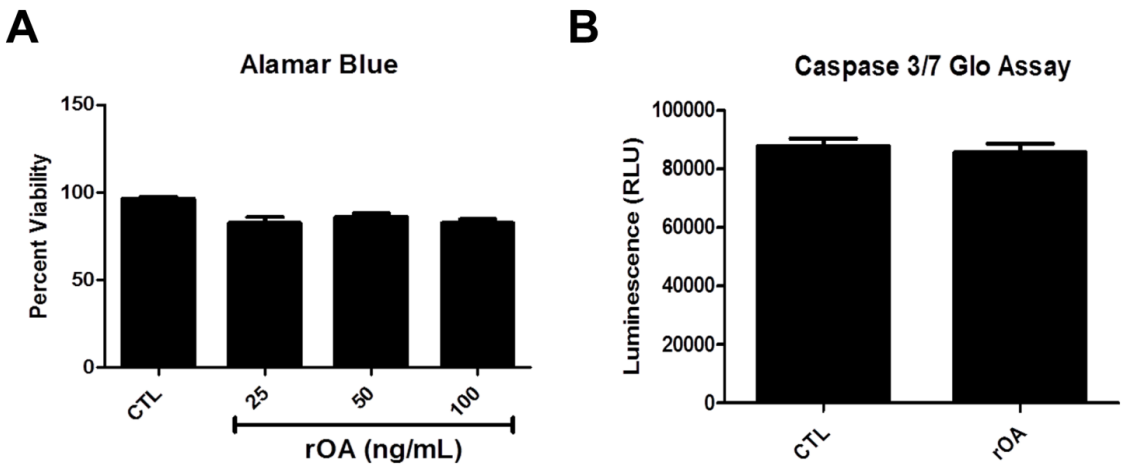


Figure 4.3: Recombinant Osteoactivin has no effect on MC3T3-E1 cell viability. (A) Alamar Blue analysis of MC3T3-E1 cells plated in 96 well plates and serum starved overnight prior to the addition of different concentrations of recombinant Osteoactivin for 48 hours. Mitochondrial activity and cell viability/apoptosis in cells untreated (control;CTL) and treated with recombinant Osteoactivin was determined by spectrophotometry. (**B**) Caspase-Glo 3/7 activity of MC3T3-E1 cells plated in 96 well plates. Briefly, cells were serum starved overnight and either left untreated (control; CTL) or treated with recombinant Osteoactivin (50ng/mL) for 48 hours. Luminescent activity was determined using a luminometer. The experiments were run in triplicates. Data presented in all graphs represent Mean ± SEM.

4.3 Osteoactivin Stimulates Osteoblast Matrix Deposition and Mineralization

In previous studies, loss of function of Osteoactivin through the use of blocking antibodies resulted in the inhibition of osteoblast differentiation [2]. In the present study, we wanted to determine if recombinant Osteoactivin stimulated osteoblast differentiation and function *in vitro*. Recombinant Osteoactivin added during osteogenic differentiation with β-Glycerophosphate and Ascorbic Acid resulted in a substantial increase in Alkaline phosphatase (ALP) staining in both C57Blk6 (WT) MSCs (Figure 4.4A) and MC3T3-E1 osteoblast like cells (Figure 4.4B). Furthermore, recombinant Osteoactivin treatment significantly increased ALP enzyme activity in both WT MSCs (Figure 4.4C) and MC3T3-E1 cells (Figure 4.4D). This indicates that recombinant Osteoactivin stimulates early matrix deposition *in vitro*.

Next, we saught to determine if recombinant Osteoactivin had an effect on late stage osteoblast matrix mineralization. Recombinant Osteoactivin was added to osteogenic media for 21 days and resulted in a substantial increase in matrix mineralization in both WT MSCs (Figure 4.5A) and MC3T3-E1 cells (Figure 4.5B). This indicates that recombinant Osteoactivin stimulates both matrix deposition and matrix mineralization during osteoblast differentiation.

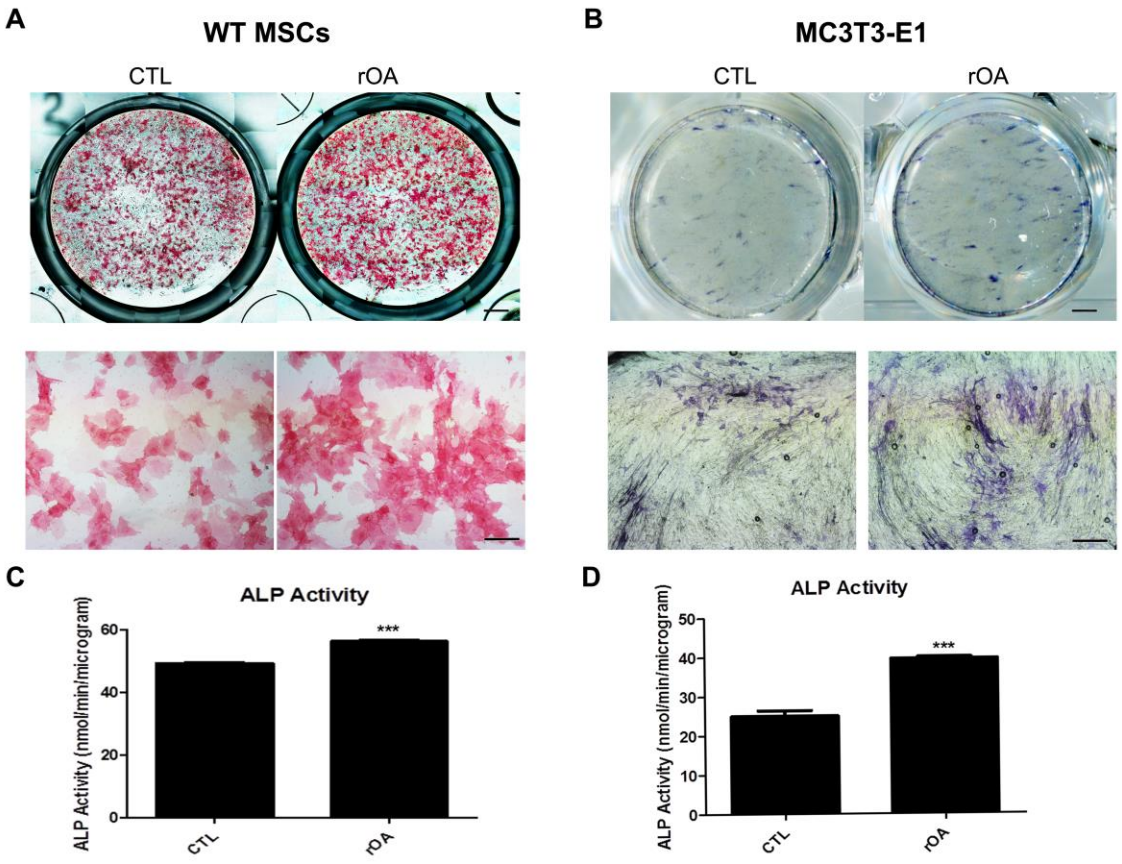


Figure 4.4: Recombinant Osteoactivin enhances osteoblast matrix deposition in both MSCs and MC3T3-E1 cells. (A-B) ALP staining in C57Blk6 (WT) MSCs (**A**) and MC3T3-E1(**B**) cells treated with recombinant Osteoactivin and osteogenic differentiation factors β-Glycerophosphate (10mM) and Ascorbic Acid (50µg/mL) or osteogenic differentiation factors alone (CTL). Briefly, 100K cells pre well were plated in 24 well plates and cultured for 7 days before termination for ALP staining. (**C-D**) ALP activity in WT MSCs (**C**) and MC3T3-E1 (**D**) cells. Briefly, day 7 differentiated cell lysates from untreated and recombinant Osteoactivin treated cells were used to determine the activity levels of ALP using a spectrophotometer. The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean ± SEM. ***=p<0.001. Scale bars: 1,000µm (A,B Top panel) and 250µm (A,B Bottom panel).

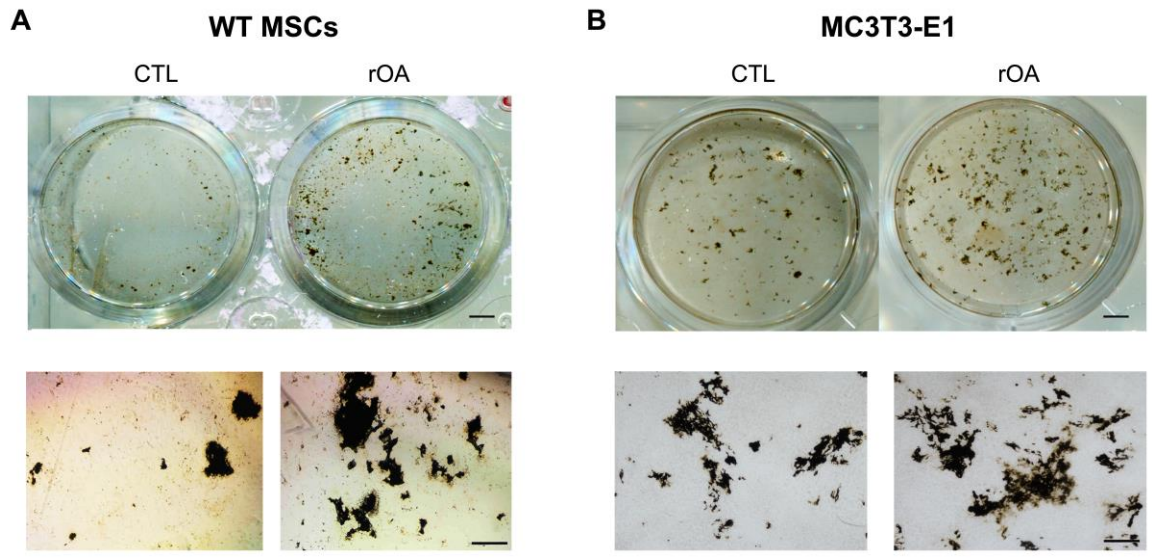


Figure 4.5: Recombinant Osteoactivin enhances osteoblast matrix mineralization in both MSCs and MC3T3-E1 cells. (A-B) Von kossa staining in WT MSCs (**A**) and MC3T3-E1 (**B**) cells treated with recombinant Osteoactivin and osteogenic differentiation factors β-Glycerophosphate (10mM) and Ascorbic Acid (50µg/mL) or osteogenic differentiation factors alone (CTL). Briefly, 100K cells per well were plated in 24 well plates and cultured for 21 days before termination for Von kossa staining. Notice the increase in mineral deposition in Osteoactivin treated samples. The experiment was repeated 3 times with similar results. Scale bars: 1,000µm (A,B Top panel) and 250µm (A,B Bottom panel).

4.4 Osteoactivin Stimulates MSC and MC3T3-E1 Osteoblast Related Gene Expression

There are several osteoblast markers that have been shown to be upregulated during osteoblast differentiation. In this study, we wanted to determine the effect of recombinant Osteoactivin on osteoblast related gene expression during the course of osteoblastogenesis. The addition of recombinant Osteoactivin (50ng/mL) to WT MSCs resulted in a significant increase in osteoblast related genes Runx2 (Figure 4.6A), Osterix (Osx) (Figure 4.6B), ALP (Figure 4.6C), and Osteocalcin (OCN) (Figure 4.6D). A similar trend was also seen in MC3T3-E1 osteoblast like cells as recombinant Osteoactivin was shown to significantly upregulate osteoblast specific genes Runx2 (Figure 4.7A), Osterix (Figure 4.7B), ALP (Figure 4.7C), and Osteocalcin (Figure 4.7D). These results indicate that Osteoactivin is able to increase osteoblast differentiation by upregulating osteoblast related markers.

As previously described, Osterix is a critical transcription factor necessary for osteoblast differentiation. Our data indicates that recombinant Osteoactivin significantly upregulates Osterix expression by qPCR. To further elaborate on this idea, we transfected MC3T3-E1 cells with the full length -1269/+91 Osterix luciferase promoter and treated the cells with recombinant Osteoactivin. MC3T3-E1 cells transfected with Osterix luciferase vector and treated with recombinant Osteoactivin showed a significant upregulation of Osterix promoter activity compared to MC3T3-E1 cells transfected with control vector (Figure 4.8). This indicates that Osteoactivin may play a role in the regulation of Osterix promoter activity and expression.

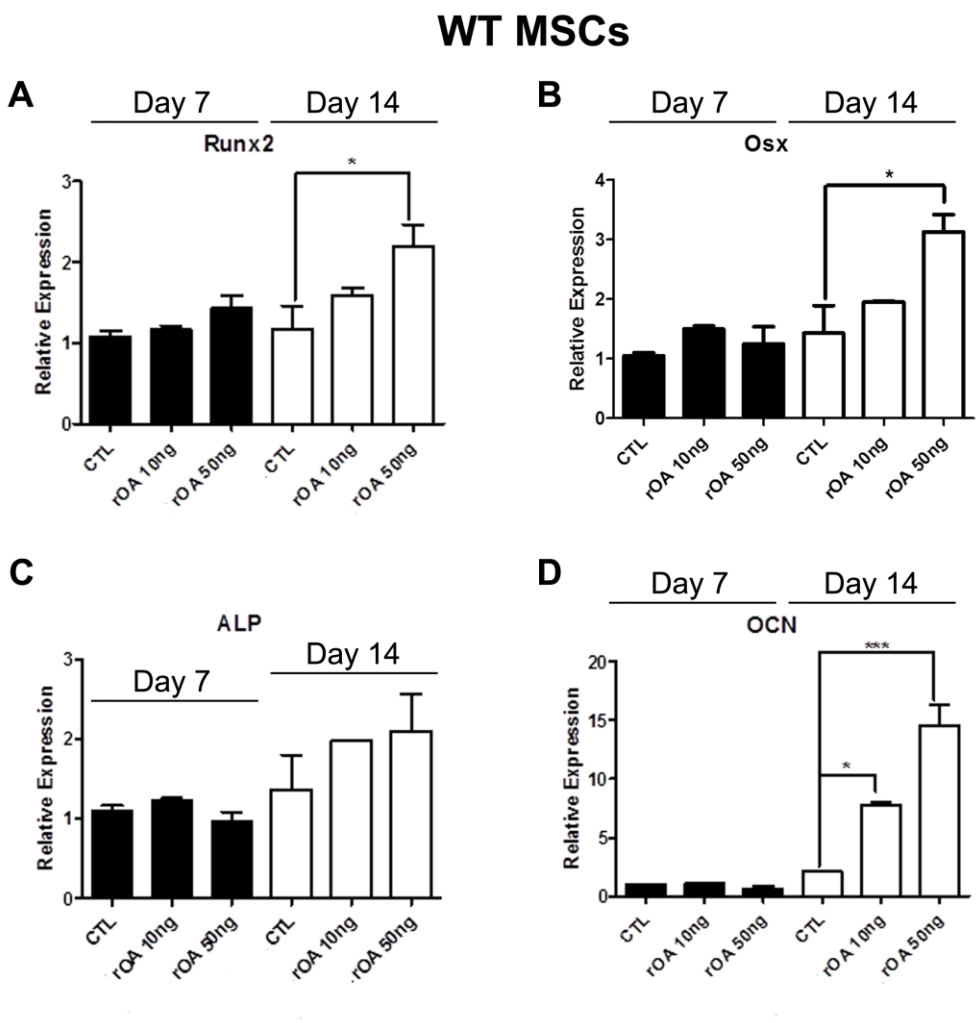


Figure 4.6: Recombinant Osteoactivin stimulates osteoblast related genes in WT MSCs during osteogenic differentiation. (**A-D**) qPCR analysis of osteoblast related genes in WT MSCs treated with different concentrations of recombinant Osteoactivin and differentiation factors or differentiation factors alone (CTL). WT MSCs were cultured with or without Osteoactivin for 7 and 14 days prior to termination. Osteoactivin upregulated several important factors important for osteoblast differentiation that include Runx2 (**A**), Osterix (Osx) (**B**), ALP (**C**), and Osteocalcin (OCN) (**D**). The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean ± SEM. ***=p<0.05, ****=p<0.001.

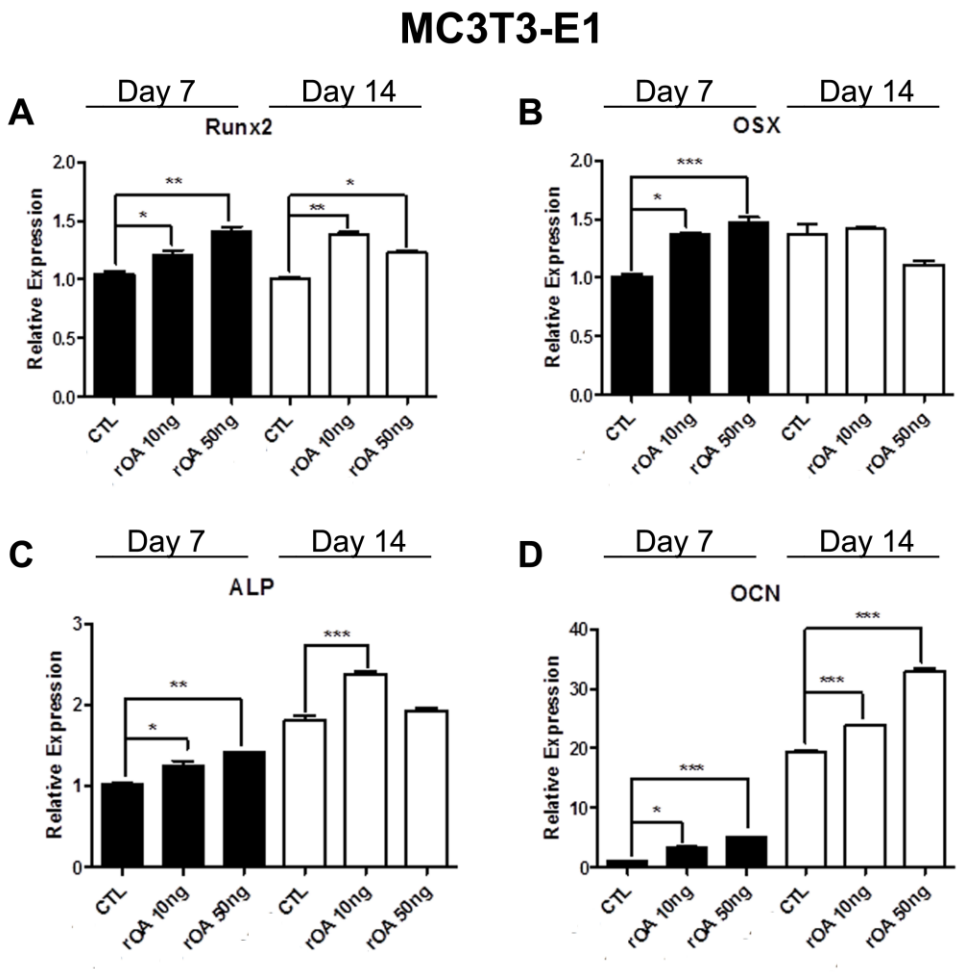


Figure 4.7: Recombinant Osteoactivin stimulates osteoblast related genes in MC3T3-E1 osteoblast like cells during osteogenic differentiation. (A-D) qPCR analysis of osteoblast related genes in MC3T3-E1 cells treated with different concentrations of recombinant Osteoactivin and differentiation factors or differentiation factors alone (CTL). Osteoactivin upregulated several important factors important for osteoblast differentiation that include Runx2 (**A**), Osterix (Osx) (**B**), ALP (**C**), and Osteocalcin (OCN) (**D**). The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean ± SEM. *****=p<0.05, ******=p<0.01, *******=p<0.001.

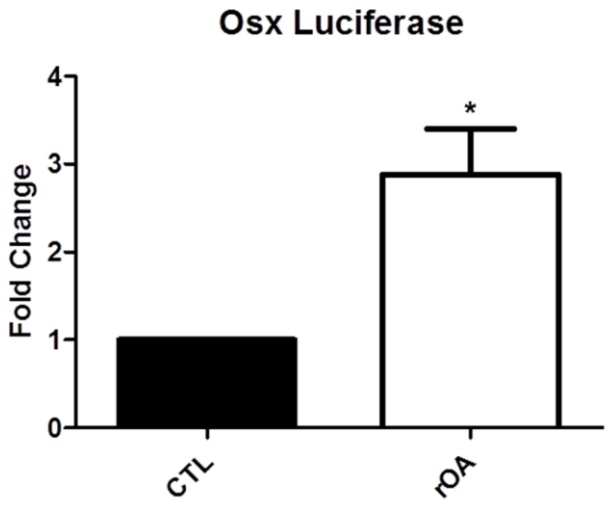
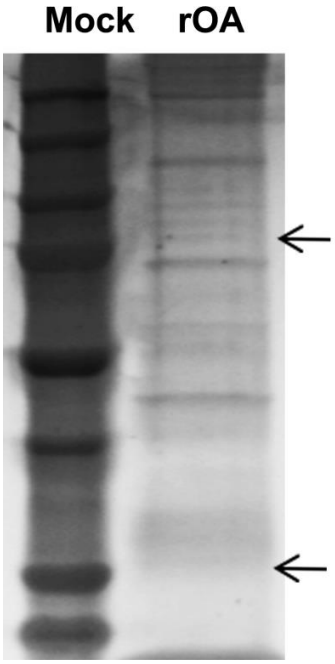


Figure 4.8: Osteoactivin regulates Osx promoter activity in MC3T3-E1 cells. Osteoblasts were seeded at 200K cells per well in 6 well plates. After 24 hours, cells were transiently transfected with either PGL3 control empty vector or the -1269/+91 Osx full length promoter reporter. 48 hours after transfection, cells were serum starved for 8 hours followed by treatment with either recombinant Osteoactivin (50ng/mL) or left untreated for 24 hours. Following treatment, cells were harvested and assayed using firefly luciferase and *Renilla* luciferase substrates with the Dual Luciferase assay system. Luciferase values were normalized to *Renilla* luciferase data to correct for variation in transfection efficiency. The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean ± SEM. *****=$p<0.05$.

4.5 Osteoactivin Binds to and Interacts with CD44 in Osteoblasts

There is very little literature describing the signaling pathway of Osteoactivin in bone cells. In order to determine a novel receptor for Osteoactivin, we biotinylated recombinant Osteoactivin and added it to MC3T3-E1 cells. The isolated protein was purified with streptavidin, and mass spectroscopy analysis was performed. Mass spectroscopy analysis identified several proteins that bound to and interacted with Osteoactivin. Among these identified proteins, CD44 and Caveolin-1 were found to have a significant interaction with recombinant Osteoactivin (Figure 4.9).

To confirm the CD44-Osteoactivin interaction, we immunoprecipitated biotinylated recombinant Osteoactivin treated cells with a CD44 antibody and analyzed samples by Western blot. Immunoprecipitation analysis revealed that Osteoactivin interacts with CD44 (Figure 4.10). Furthermore, immunofluorescent analysis revealed co-localization of CD44 and Osteoactivin along the osteoblast cell membrane (Figure 4.11). This indicates that Osteoactivin may interact and bind to CD44. Further information is needed on the role of CD44 in bone cells.



Identified proteins from Mass Spectroscopy	Score
CD44 Antigen	108
Caveolin-1	105

Figure 4.9: Streptavidin affinity purification of *N*-hydroxysuccinimidbiotin (NHS-Biotin) labeled recombinant Osteoactivin. MC3T3-E1 cells were treated with NHS-Biotin labeled recombinant Osteoactivin for 30 minutes. Following treatment, protein was isolated and subjected to streptavidin affinity purification. After the purification, NHS-Biotin labeled recombinant Osteoactivin proteins were separated by 10% SDS-PAGE and silver stained to visualize bands. The bands were subjected to Nano-LC/MS/MS analysis to determine potential receptors for recombinant Osteoactivin. The stain identifies multiple bands, however, analysis revealed CD44 (top arrow) and Caveolin-1(bottom arrow) as potential receptors for Osteoactivin.

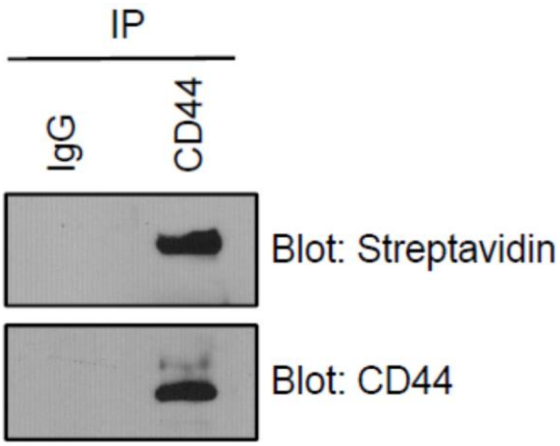


Figure 4.10: Biotinylated recombinant Osteoactivin binds to CD44 in MSCs. MSC cell lysates treated with biotinylated recombinant Osteoactivin were isolated and immunoprecipitated (IP) with either IgG (control) or anti-CD44 antibodies for 1 hour at 4^{o}C. Following immunoprecipitation, proteins were subjected to Western blot for Streptavidin (53kDa) and CD44 (80kDa).

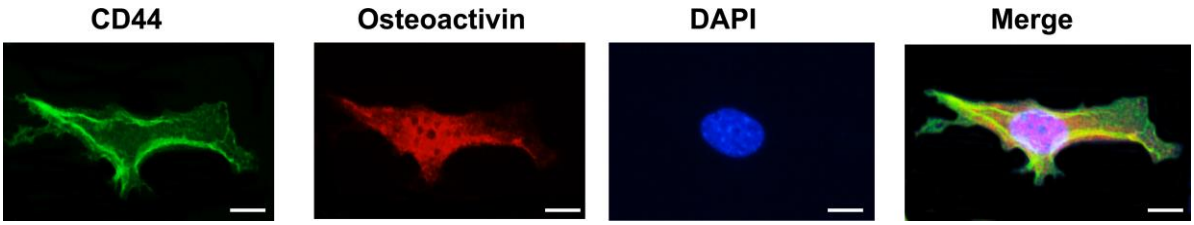


Figure 4.11: CD44 and Osteoactivin colocalize at the cell membrane in MC3T3-E1 cells. Immunoflourecent analysis of the localization of CD44 and Osteoactivin. MC3T3-E1 cells were seeded at a density of 10K cells per well and cultured in chamber slides. Cells were fixed, stained with anti-CD44 or anti-Osteoactivin, and counterstained with DAPI in order to visualize the localization of these molecules in MC3T3-E1 cells. Merged images of CD44 (green), Osteoactivin (red), and DAPI (blue) were generated in order to visualize colocalization of CD44 and Osteoactivin along the cell membrane. Scale bars: 10μm

4.6 CD44 Deficient Mice have Significantly Less Bone Mass in vivo

The first report of CD44 expression in bone came from a study that revealed that CD44 is important for osteocytes and osteocyte differentiation [385]. Several studies have shown that CD44 is highly expressed in MSCs, osteoblasts, and osteoclasts [386, 393, 447]. Previous studies have also shown that CD44 may have anti-inflammatory properties in both bone and cartilage [391, 448]. Therefore, in order to understand the role of CD44 in bone homeostasis, we characterized the skeletal phenotype of CD44 deficient mice (CD44KO) both *in vivo* and *ex vivo*. MicroCT analysis revealed a dramatic reduction in bone mass at both 8 and 16 week male mice (Figure 4.12). The reduction in the bone volume/tissue volume (BV/TV) seen in CD44 deficient mice was approximately 30% less compared to wild type (WT) controls at 8 weeks of age and approximatley 20% less at 16 weeks of age (Figure 4.13A). Bone area (B.Ar), bone perimeter (B.Pm), and trabecular number (Tb.N) were all significantly less in CD44 deficient mice at both 8 and 16 weeks compared to WT(Figure 4.13B-D). Trabecular spacing was significantly more in CD44 deficient mice compared to wild type at both 8 and 16 weeks (Figure 4.13E). The trabecular thickness (Tb.Th) was also slightly decreased in CD44 deficient mice at 8 weeks but revealed no significant difference at 16 weeks (Figure 4.13F). Several of the differences in the microCT parameters observed between the 8 week and 16 week mice were more pronounced in the 8 week age mice. This may indicate that the defect in bone homeostasis observed in CD44 deficient mice may happen at an earlier age and that during the remodeling phase the bone phenotype may begin to correct itself.

To further elaborate on the bone phenotype of the CD44 deficient mice *in vivo*, we performed histomorphometric analyses on 8 week WT and CD44 deficient mice. Von kossa staining revealed a significant reduction in mineralization in the CD44 deficient mice (Figure 4.14). Histomorphometric analysis of CD44 deficient mice revealed a significant reduction in trabecular number (Tb.N), bone perimeter/total area (B.Pm/T.Ar), and trabecular number/total area (Tb.N/T.Ar) and an increase in trabecular separation (Tb.Sp) compared to WT (Figure 4.15A-D). Interestingly, there seemed to be no difference in the number of osteoblasts per bone perimeter (N.Ob/B.Pm) or the percentage of osteoblasts per bone surface (Ob.S/B.S.) (Figure 4.15E-F). This indicates that the reduction in bone mass in CD44 deficient mice may be due to defective osteoblast differentiation and function independent of osteoblast proliferation and number.

To further investigate the kinetic rate of bone formation *in vivo* in CD44 deficient mice, we performed calcein labeling and histmorphometric analysis. CD44 deficient mice had approximately a 30% reduction in mineral apposition rate (MAR) and bone formation rate (BFR) (Figure 4.16A-C). Taken together, these data indicate that CD44 deficient mice have impaired osteoblasts which results in defective mineralization and bone formation *in vivo*.

Next, we performed serum analysis for osteoblast and osteoclast markers in both WT and CD44 deficient mice at 8 and 16 weeks by ELISA to further understand the bone phenotype *in vivo*. At 8 weeks age, osteoblast markers P1NP and Osteocalcin were significantly reduced in CD44 deficient mice; however, at 16 weeks, only Osteocalcin was significantly reduced (Figure 4.17A-B). This may be best explained as

an age specific bone phenotype where there seems to be more of an impairment in osteoblasts at 8 weeks compared to 16. Furthermore, a compensatory mechanism may start to take place resulting in the bone phenotype beginning to correct itself at 16 weeks. Osteoclast serum analysis markers RANKL, OPG, and CTX-1 showed no significant differences between WT and CD44 deficient mice (Figure 4.18A-D). This indicates that the loss of bone mass in the CD44 deficient mice is primarily due to the osteoblasts, not the osteoclasts.

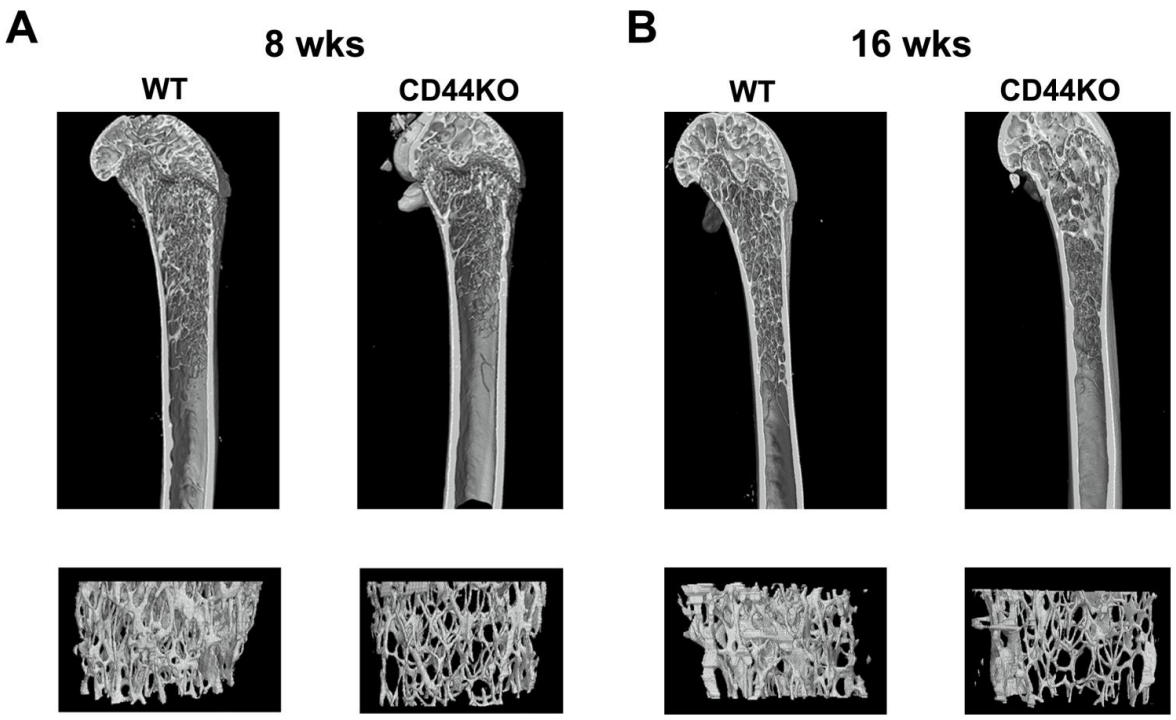


Figure 4.12: CD44 deficient mice have significantly less bone mass. (**A-B**) MicroCT three-dimensional reconstructed images of C57Blk6 (WT) and CD44 deficient (CD44KO) femurs in 8 week (**A**) and 16 week (**B**) mice. Mouse femurs (n=6 per group) were analyzed using a SkyScan high-resolution microtomography system. Three-dimensional images of the sagittal planes of whole femurs (top panel) and femoral metaphysis (bottom panel) are shown.

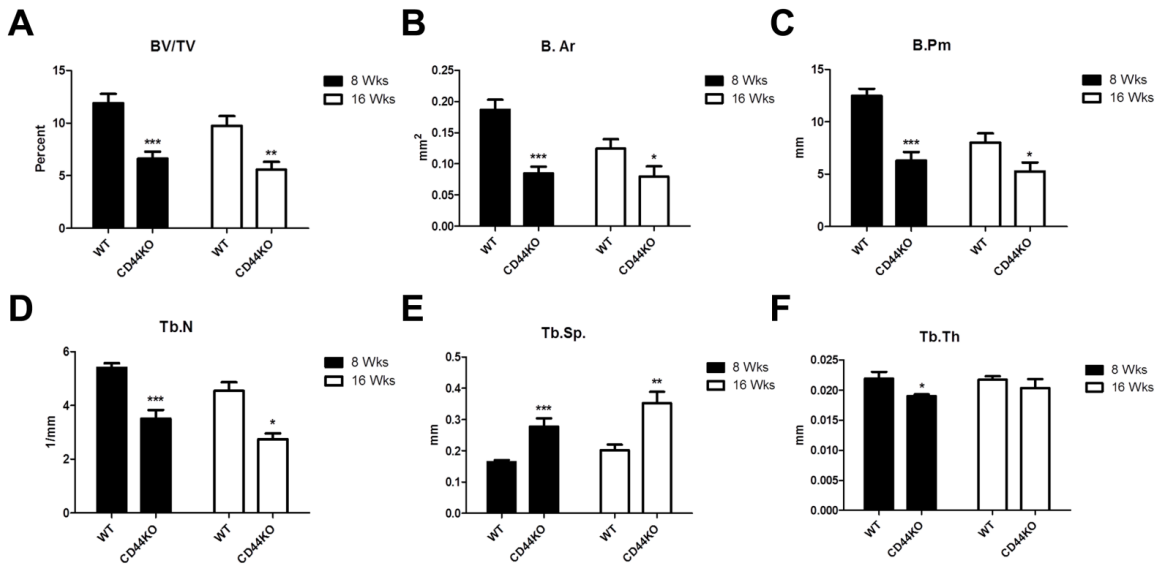


Figure 4.13: CD44 deficient mice have significantly less bone parameters. (**A-F**) Quantification of microCT analysis of WT and CD44KO femurs (n=6 per group) in both 8 week and 16 week old mice for BV/TV (**A**), B.Ar (**B**), B.Pm (**C**), Tb.N (**D**), Tb. Sp. (**E**), and Tb.Th (**F**). CD44 deficient mice had significantly less BV/TV, B.Ar, B.Pm, Tb.N, Tb.Th, but more Tb.Sp. compared to WT. Notice the dramatic difference at 8 weeks compared to 16 weeks. Data presented in all graphs represent Mean ± SEM. *****=p<0.05, ******=p<0.01, *******=p<0.001.

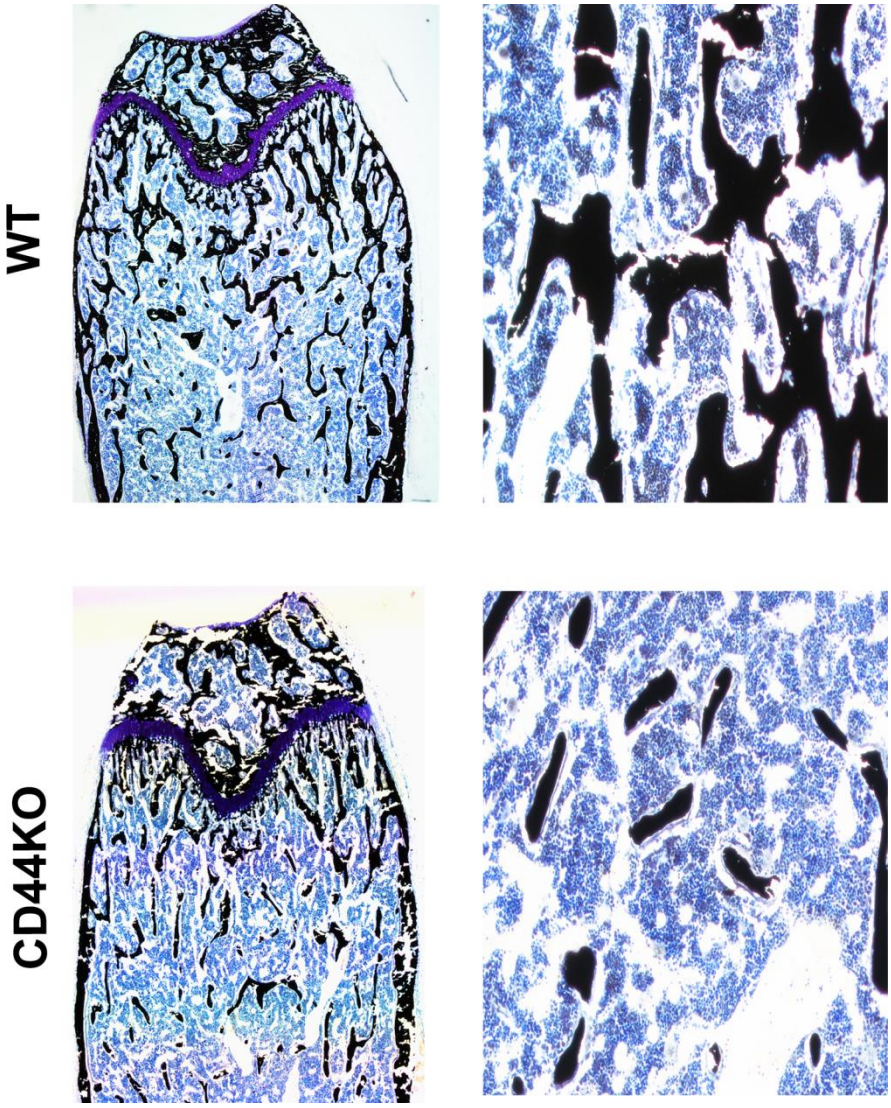


Figure 4.14: CD44 deficient mice have significantly less mineralization *in vivo*.

Saggital sections from WT and CD44KO 8 week old mouse femurs (n=4) were sectioned and stained with Von kossa to visualize minerals (black) and counterstained with toluidine blue. Sections of the distal femur were imaged at 2X (left) and 10X (right) magnification.

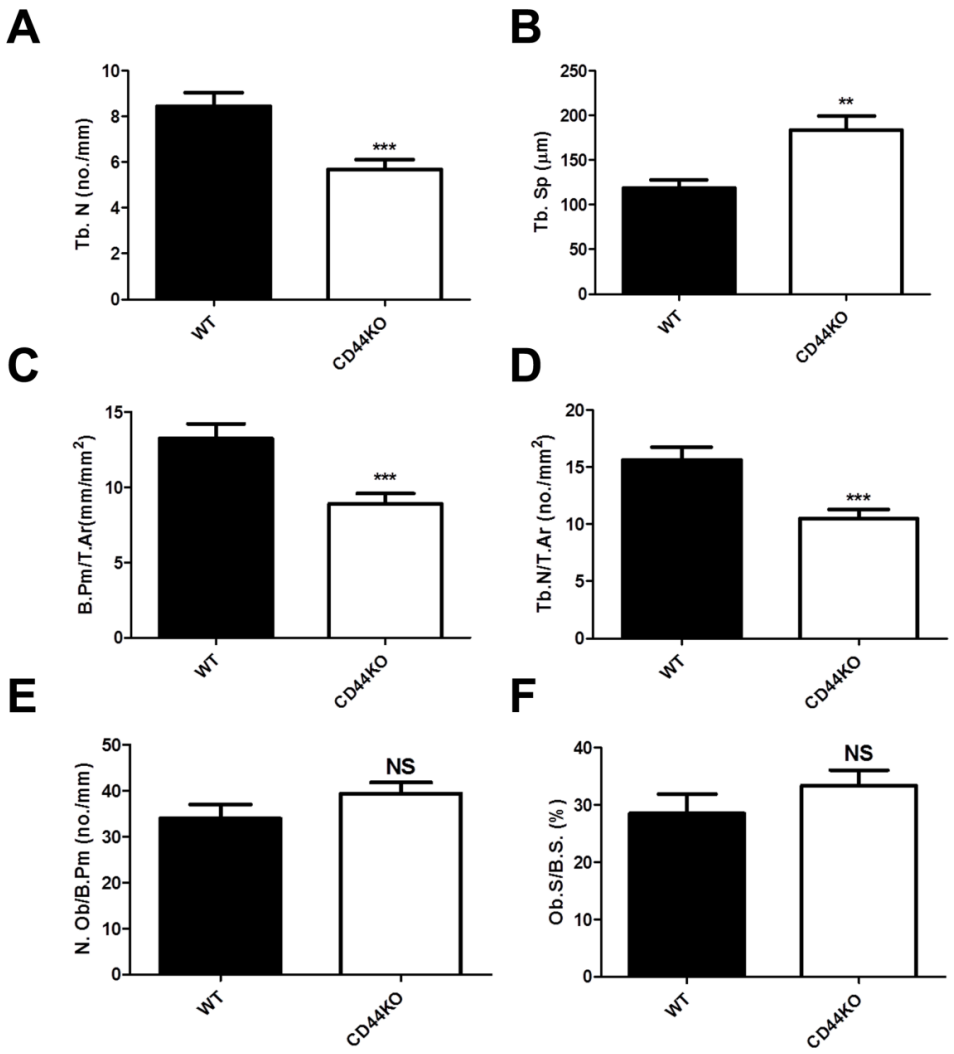


Figure 4.15: Quantitative histomorphometric analysis reveals significantly decrease bone mass but no difference in osteoblast number. Sagittal sections from 8 week old WT and CD44KO mouse femurs and were stained for Von kossa. (**A-F**) Histomorphometric parameters including Tb.N (**A**), Tb. Sp (**B**), B.Pm/T.Ar (**C**), and Tb.N/T.Ar (**D**) were all significantly different in CD44KO compared to wild type mice, however, N. Ob./B.Pm (**E**) and Ob.S/B.S. (**F**) showed no difference. Data presented in all graphs represent Mean ± SEM. **=$p<0.01$, ***=$p<0.001$.

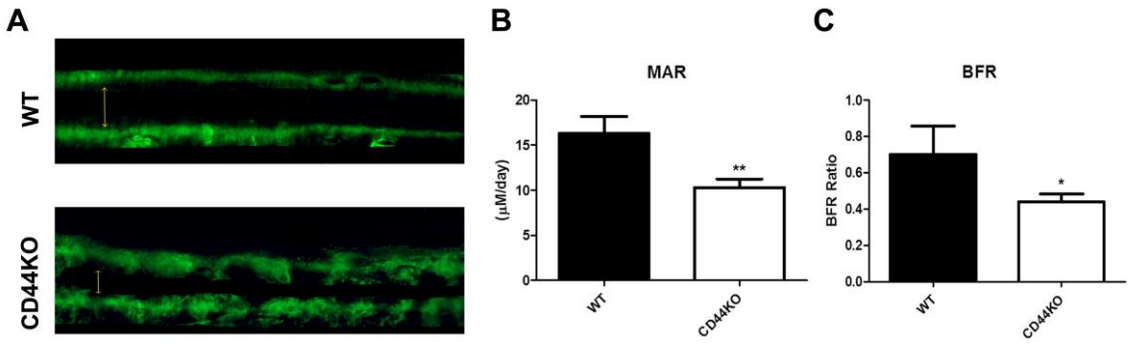


Figure 4.16: CD44 deficient mice have a reduction in mineral apposition and bone formation rate *in vivo*. (**A**) WT and CD44KO 8 week old mice were injected with Calcein AM to label the mineralized bone surface (green). After termination, unstained sagittal sections were visualized under epiflourescence to observe and quantify kinetic histomorphometric parameters MAR (**B**) and BFR (**C**). Data presented in all graphs represent Mean ± SEM. *****=p<0.05, ******=p<0.01.

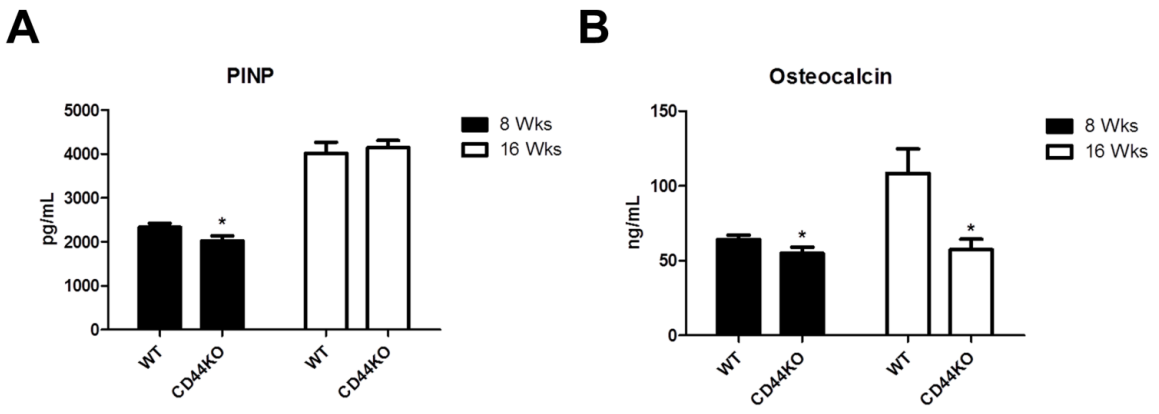


Figure 4.17: CD44 deficient mice have significantly less serum biomarkers for bone formation *in vivo*. (**A-B**) Biochemical analysis of osteoblast specific markers PINP (**A**) and Osteocalcin (**B**) in serum from 8 and 16 week old WT and CD44KO mice. Data presented in all graphs represent Mean ± SEM. *****=$p<0.05$.

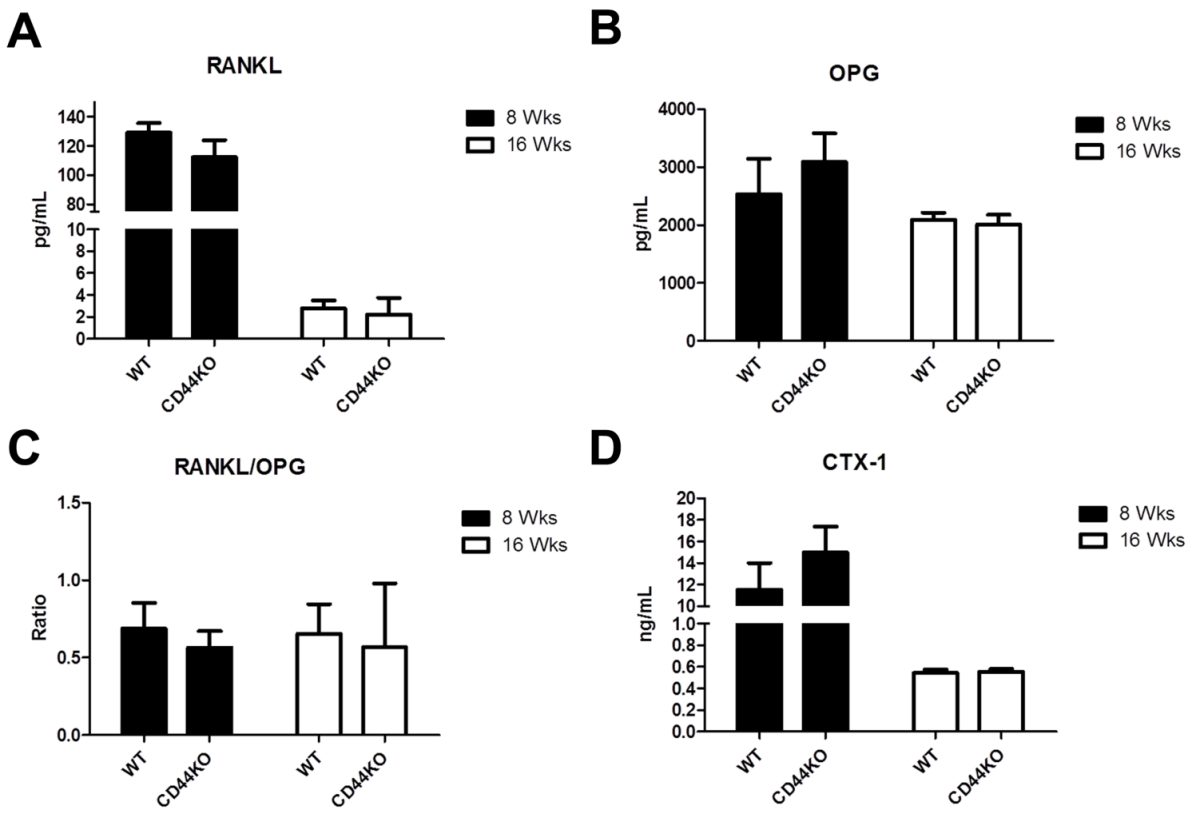


Figure 4.18: CD44 deficient mice reveal no significant difference in osteoclast serum biomarkers compared to WT *in vivo*. (**A-D**) Biochemical analysis of osteoclast serum biomarkers RANKL (**A**), OPG (**B**), ratio of RANKL/OPG (**C**), and CTX-1 (**D**) from 8 and 16 week old WT and CD44KO mice.

4.7 CD44 Deficient Mice have Significantly Less Bone ex-vivo due to Defective Osteoblast Differentiation and Function

In order to explain the low bone mass phenotype observed in the CD44 deficient mice *in vivo*, we isolated bone marrow derived mesenchymal stem cells from both WT and CD44 deficient mice and cultured them *ex vivo*. The presence or absence of CD44 in both WT and CD44 deficient cells respectively, was determined by both qPCR and Western blot (Figure 4.19). In order to determine the effect of the absence of CD44 on osteoblast differentiation, we plated WT and CD44 deficient MSCs and treated them with osteogenic factors for 7 and 14 days. QPCR analysis revealed a significant reduction in the levels of Runx2 (Figure 4.20A), Osterix (OSX) (Figure 4.20B), ALP (Figure 4.20C), and Osteocalcin (OCN) (Figure 4.20D) in the CD44 deficient mice compared to WT. This indicates that the absence of CD44 inhibits osteogenic gene expression and that CD44 may play an important role during osteoblast differentiation

To further elaborate on the role of CD44 during osteoblast differentiation, we isolated MSCs from WT and CD44 deficient mice and differentiated them in culture for 7 and 21 days in order to determine the role of CD44 in osteoblast matrix deposition and mineralization. CD44 deficient MSCs revealed significantly less ALP staining and activity compared to WT (Figure 4.21A-B). This indicates that CD44 may play a role as a positive regulator of osteoblast matrix deposition. Furthermore, CD44 deficient osteoblasts revealed significantly less mineralization by Von kossa staining (Figure 4.21C-D). These results indicate that CD44 plays a role in both osteoblast matrix deposition and mineralization, and is an overall positive regulator of osteoblast differentiation.

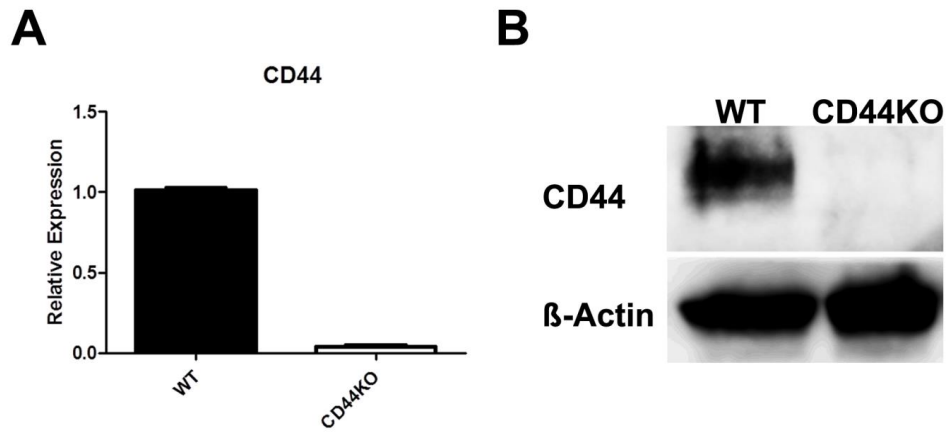


Figure 4.19: Confirmation of the absence of CD44 in CD44 deficient osteoblasts. (**A-B**) Primary osteoblasts from WT and CD44KO were plated at a density of 200K cells per well in 6 well plates. The next day, RNA and protein were isolated to determine the absence of CD44 by qPCR (**A**) and Western blot (**B**) respectively. The experiment was repeated 3 times with similar results.

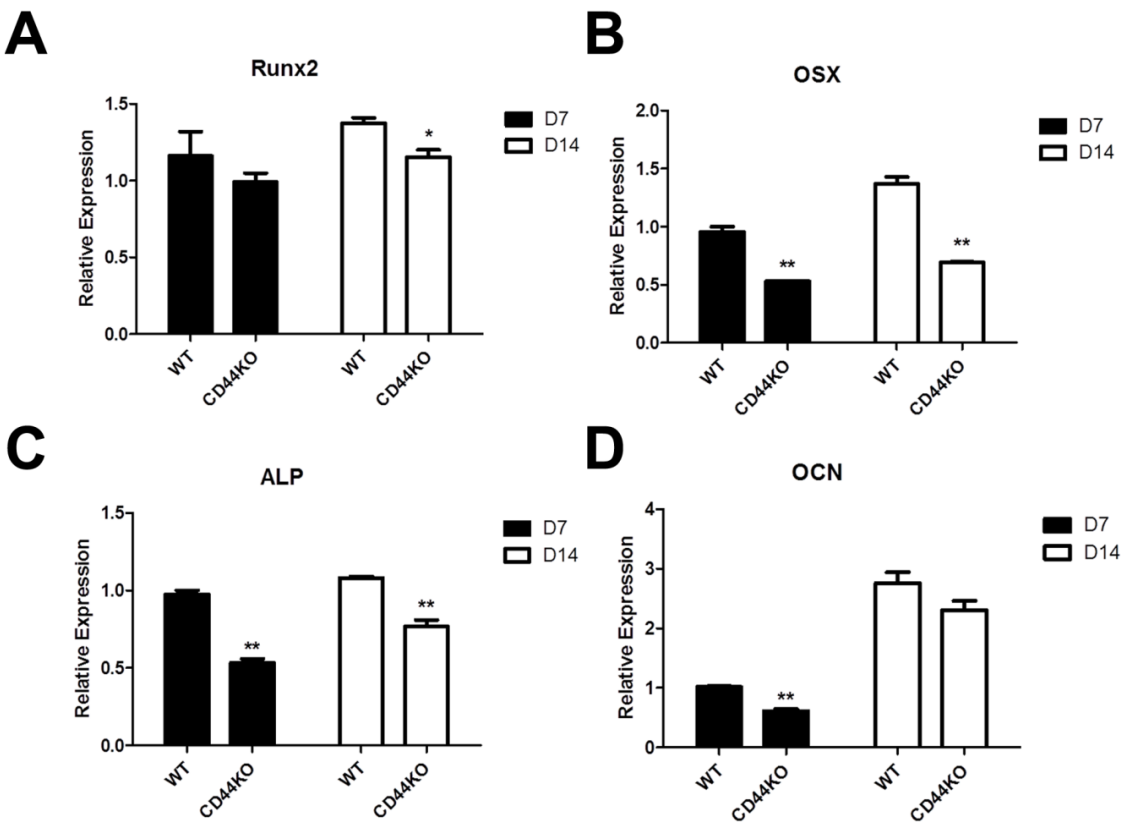


Figure 4.20: CD44 deficient MSCs show a significant reduction in osteoblast related gene expression during osteoblastogenesis. (**A-D**) qPCR analysis of mRNA from WT and CD44KO MSCs during osteoblast differentiation. MSCs taken from 8 week old WT and CD44KO male mice were plated in 6 well plates and differentiated with osteogenic factors for up to 14 days. RNA was isolated at days 7 and 14 from both WT and CD44KO MSCs and the relative expression of osteoblast related genes Runx2 (**A**), OSX (**B**), ALP (**C**), OCN (**D**) was determined by qPCR. Data presented in all graphs represent Mean ± SEM. *****=$p<0.05$, ******=$p<0.01$ compared to WT.

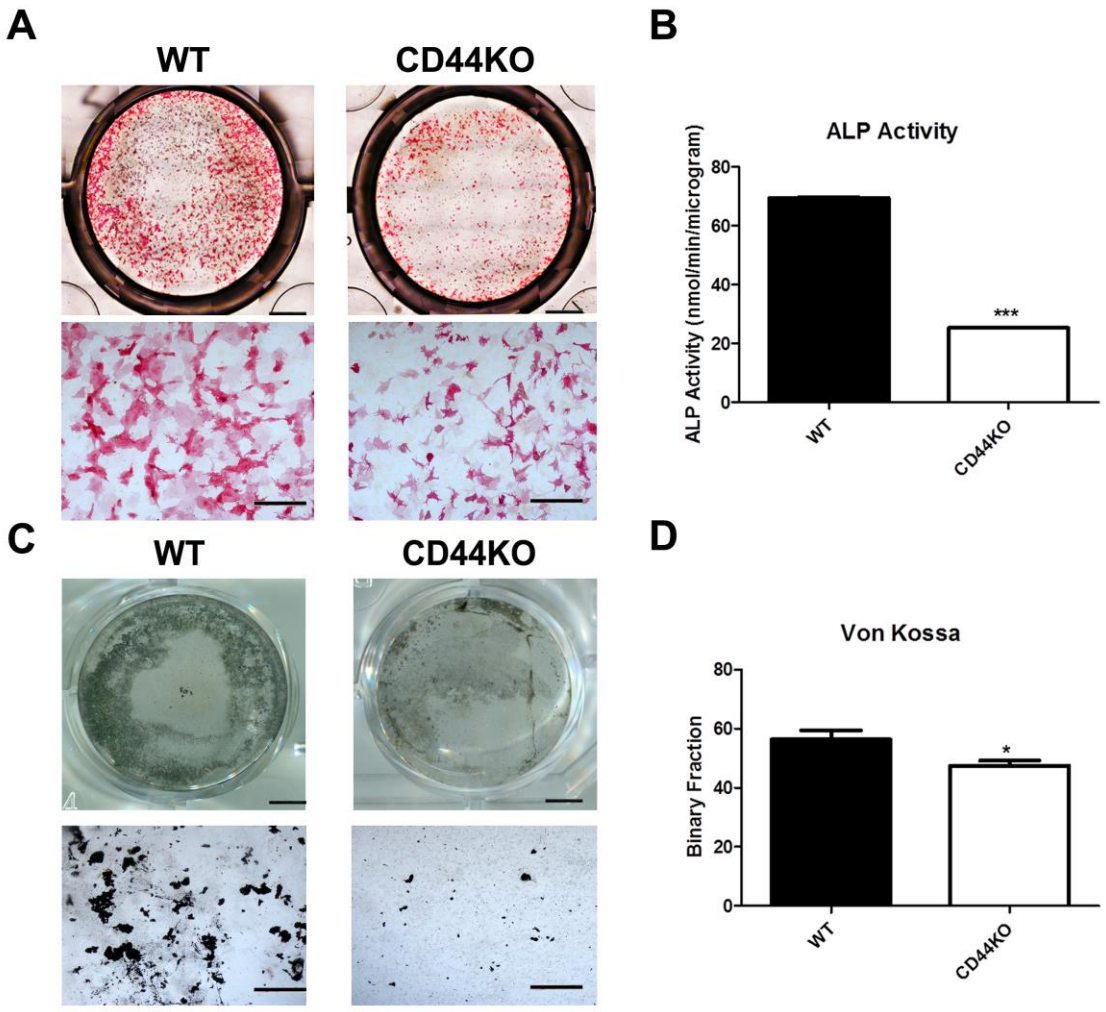


Figure 4.21: CD44 deficeint mice have significantly reduced matrix deposition and mineralization *ex vivo*. (**A-B**) WT and CD44KO MSCs were plated and differentiated with osteogenic factors for 7 days and terminated for ALP staining (**A**) and activity (**B**) in order to assess osteoblast matrix deposition. Furthermore, WT and CD44KO MSCs were plated and differentiated for 21 days and terminated for Von kossa staining (**C**) and quantitation of minerals (**D**) to assess matrix mineralization. The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean ± SEM. ***=p<0.05, ****=p<0.001 compared to WT. Scale bars: 1,000µm (Top panel) and 250µm (Bottom panel).

4.8 Osteoactivin Stimulates MAPKinase Signaling in Osteoblasts through CD44

Previous literature has indicated that Osteoactivin may play a role in the MAPK pathway in several different cell types including fibroblasts, MSCs, and osteoclasts [7, 366, 449]. In this study, we wanted to determine the effects on recombinant Osteoactivin treatment on downstream signaling pathways. Recombinant Osteoactivin treatment increased phosphorylated ERK signaling in a dose dependent and time dependent manner (Figure 4.22A-B). A similar response was seen in phosphorylated P38 (Figure 4.23A-B) but not phosphorylated JNK (data not shown). This indicates that recombinant Osteoactivin plays a role in stimulating certain members of the MAPK pathway.

In order to further characterize the Osteoactivin mediated signaling pathway, we transfected MC3T3-E1 cells with CD44 siRNA and treated cells with recombinant Osteoactivin and observed activation of ERK and P38 activation. MC3T3-E1 cells transfected with CD44 and treated with recombinant Osteoactivin revealed a significant reduction in both ERK and P38 phosphorylation compared to cells treated with recombinant Osteoactivin alone (Figure 4.24A-C). This indicates that Osteoactivin stimulates ERK and P38 signaling through the CD44 receptor in osteoblasts.

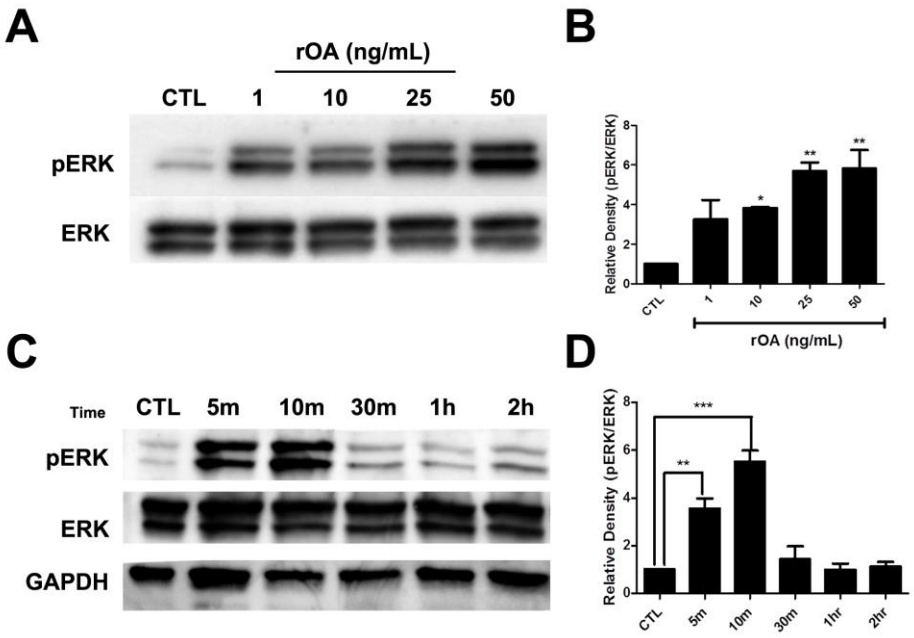


Figure 4.22: Recombinant Osteoactivin stimulates phosphoERK in osteoblasts in a dose and time dependent manner. (**A-B**) Western blot analysis of MC3T3-E1 cells treated with different concentrations of recombinant Osteoactivin. Briefly, MC3T3-E1 cells were plated at 200K cells per well in 6 well plates and serum starved overnight. Cells were then treated for 15 minutes with different concentrations of recombinant Osteoactivin and protein was isolated and analyzed by Western blot. Membranes were probed with phosphorylated and total ERK. Western blot images (**A**) and densitometric analysis (**B**) revealed a significant increase in phosphorylated ERK with increasing concentrations of recombinant Osteoactivin. (**C-D**) Western blot analysis of MC3T3-E1 cells treated with recombinant Osteoactivin. MC3T3-E1 cells were serum starved overnight and treated with 50 ng/mL recombinant Osteoactivin and terminated at different time points. The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean ± SEM. *****=p<0.05, ******=p<0.01, *******=p<0.001 compared to untreated control samples.

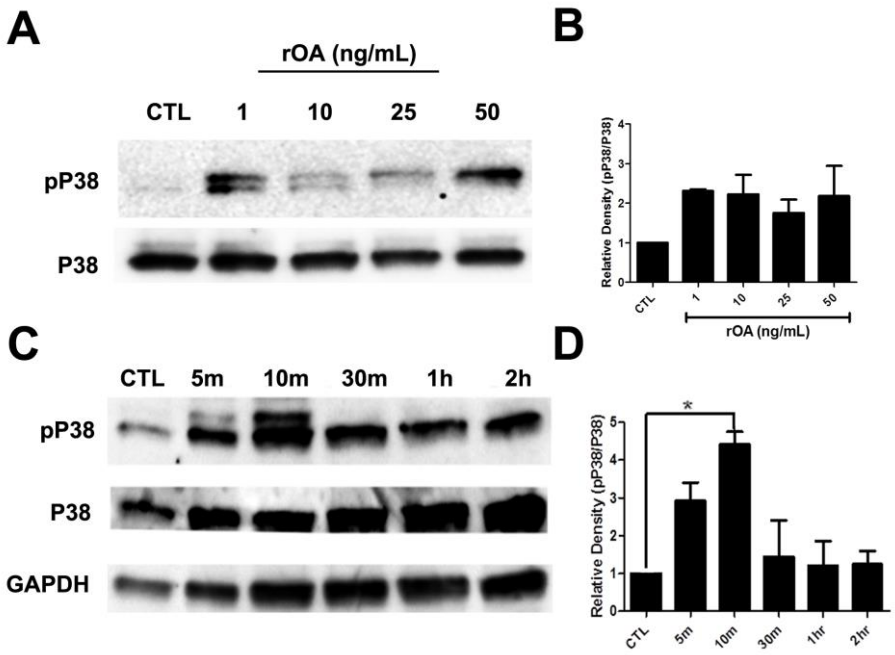


Figure 4.23: Recombinant Osteoactivin stimulates phosphoP38 in osteoblasts in a dose and time dependent manner. (**A-B**) Western blot analysis of MC3T3-E1 cells treated with recombinant Osteoactivin with different concentrations as indicated in the figure. Briefly, MC3T3-E1 cells were plated in 6 well plates at a seeding density of 200K cells per well and serum starved overnight. Cells were then treated for 15 minutes with different concentrations of recombinant Osteoactivin and protein was isolated and analyzed by Western blot. Membranes were probed with phosphorylated and total P38. Western blot images (**A**) and densitometric analysis (**B**) revealed a significant increase in phosphorylated P38 with increasing concentrations of recombinant Osteoactivin. (**C-D**) Western blot analysis of MC3T3-E1 cells treated with recombinant Osteoactivin. MC3T3-E1 cells were serum starved overnight and treated with 50 ng/mL recombinant Osteoactivin and terminated at different time points as indicated. The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean ± SEM. *****=$p<0.05$ compared to untreated controls.

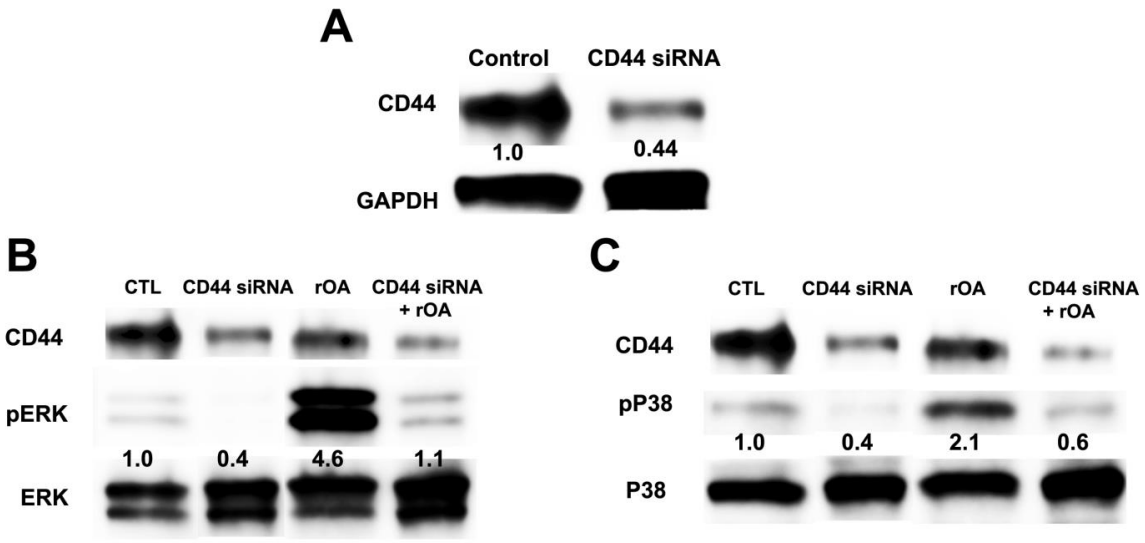


Figure 4.24: Knockdown of CD44 in MC3T3-E1 cells results in a decrease in Osteoactivin induced phosphoERK and phosphoP38. (**A**) Western blot analysis of MC3T3-E1 cells knocked down with CD44 siRNA. Briefly, cells were plated in 6 well plates at a seeding density of 200K cells per well and transfected with 100 nM of either scrambled or CD44 siRNA for 48 hours. Protein was isolated and membranes were probed with antibodies against CD44 and GAPDH to confirm knockdown of CD44. (**B-C**) Western blot analysis of MC3T3-E1 cells knocked down with CD44 siRNA and treated with recombinant Osteoactivin (50ng/mL). Briefly, cells were transfected with CD44 siRNA and either left untreated (CTL and CD44siRNA) or treated with recombinant Osteoactivin (rOA and CD44siRNA and rOA) for 10 minutes. Protein was isolated and membranes were probed for phospho and total ERK (**B**) and phospho and total P38 (**C**).

4.9 Loss of CD44 Prevents the Enhancement of Recombinant Osteoactivin Mediated Osteoblast Differentiation

As previously described, recombinant Osteoactivin stimulates ALP staining and activity in WT MSCs and that absence of CD44 results in defective osteoblast differentiation. In order to confirm that Osteoactivin mediated stimulation of MSCs is through the CD44 receptor, we differentiated MSCs from WT and CD44KO mice and either left them untreated (control;CTL) or treated with recombinant Osteoactivin for 7 days. Results from ALP staining and activity revealed that Osteoactivin stimulation of ALP staining and activity is abrogated in CD44 deficient MSCs (Figure 4.25A-B). This indicates that Osteoactivin induced stimulation of osteoblast differentiation is mediated through the CD44 receptor.

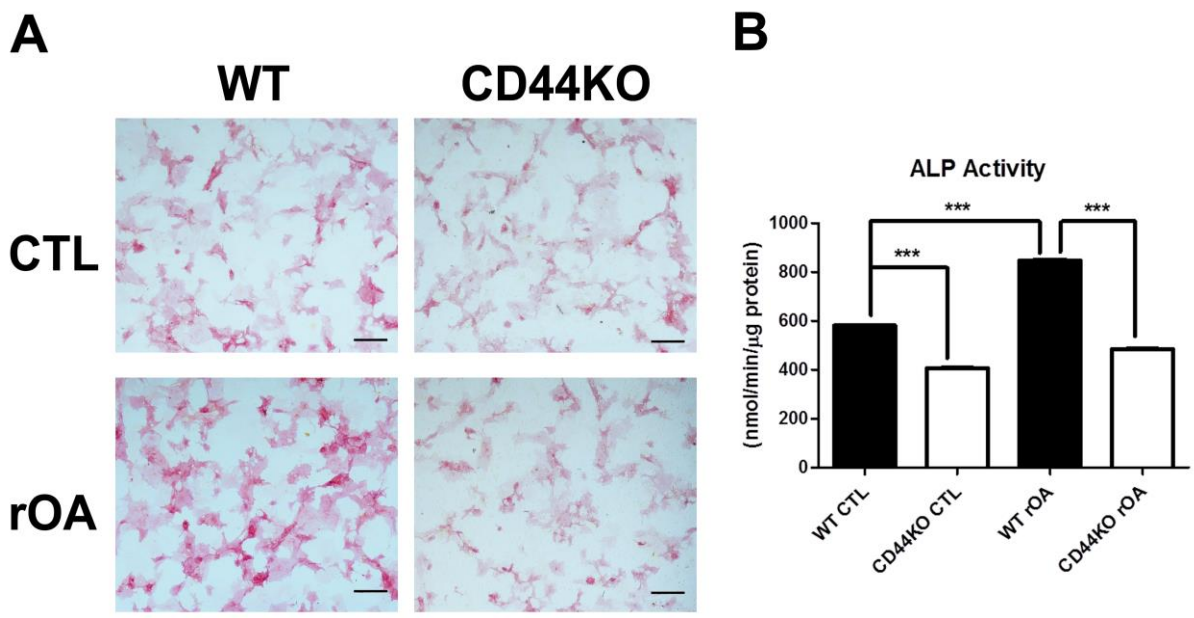


Figure 4.25: Recombinant Osteoactivin stimulation of osteoblast differentiation is abrogated in CD44 deficient MSCs. (**A-B**) WT and CD44KO MSCs were plated in 24 well plates at a seeding density of 100K cells per well and treated with either osteogenic media alone (control; CTL) or osteogenic media with recombinant Osteoactivin (50ng/mL) for 7 days. After 7 days, the cells were terminated to assess osteoblast matrix deposition by ALP staining (**A**) and activity (**B**). The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean ± SEM. ***=$p<0.001$ when compared to WT and untreated controls. Scale bars: 200 µm (Panel A)

4.10 Recombinant Osteoactivin Induces Osteoblast Differentiation through the CD44-ERK Signaling Axis

Next, we wanted to determine if Osteoactivin mediated stimulation of ERK phosphorylation through CD44 had any effect on osteoblast regulatory genes. First, primary osteoblasts isolated from WT and CD44 deficient mice were plated and either left untreated (CTL) or treated with recombinant Osteoactivin. Primary osteoblasts treated with recombinant Osteoactivin showed a significant increase in osteoblast related genes Col1α1 (Figure 4.26A) and Osterix (Figure 4.26B) in WT cells by qPCR; however, this effect was abrogated in CD44 deficient cells treated with Osteoactivin. Interestingly, recombinant Osteoactivin mediated increase of Runx2 in WT cells did not show a difference in CD44 deficient cells treated with recombinant Osteoactivin (Figure 4.26C). This indicates that Osteoactivin upregulation of Col1α1 and Osterix is mediated through CD44, and that Osteoactivin mediated stimulation of Runx2 seems to be CD44 independent.

Furthermore, in order to link the CD44-ERK signaling axis to downstream osteoblast gene expression, we transfected MC3T3-E1 cells with CD44 siRNA and treated with recombinant Osteoactivin and an ERK inhibitor U0126 alone or in combination. Cells transfected with CD44 siRNA and treated with recombinant Osteoactivin resulted in a decrease in Col1α1 and Osterix compared to recombinant Osteoactivin treated cells alone (Figure 4.27A-B). Similarly, cells treated with U0126 and Osteoactivin together also saw a reduction in Col1α1 and Osterix combined compared to Osteoactivin treated cells alone. Interestingly, cells transfected with CD44 siRNA and treated with U0126 and Osteoactivin combined resulted in an even greater

inhibition of Col1α1 and Osterix. This indicates that Osteoactivin stimulates Col1α1 and Osterix expression through the CD44-ERK signaling axis. Osteoactivin stimulation of Runx2 shows a slightly different pattern. Cells transfected with CD44 siRNA and treated with recombinant Osteoactivin showed no difference compared to Osteoactivin treated cells alone, however, when pretreated with U0126 Runx2 showed a significant reduction compared to Osteoactivin treated cells alone (Figure 4.27C). This indicates that Osteoactivin induced upregulation of Runx2 is mediated through ERK signaling, but not through CD44. Further studies of other potential receptors responsible for Osteoactivin mediated upregulation of Runx2 expression in osteoblasts should be conducted.

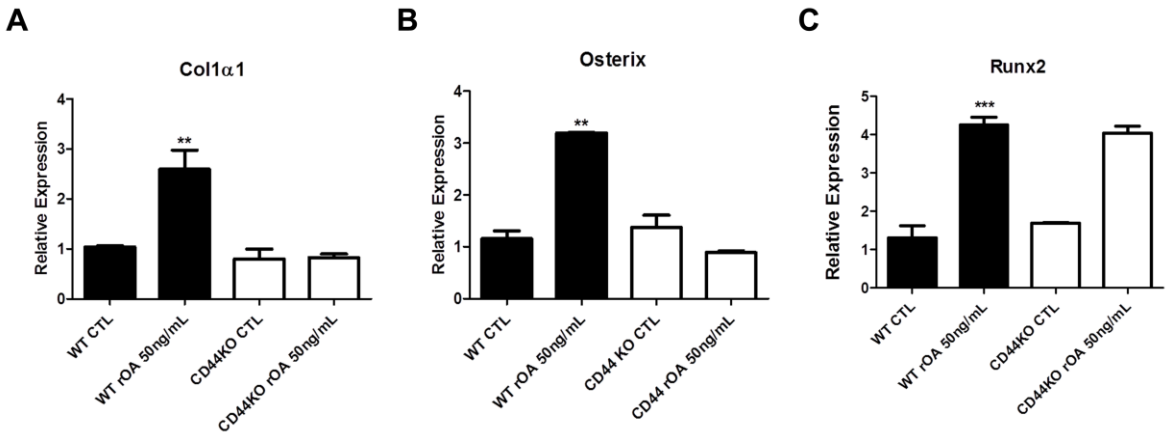


Figure 4.26: CD44 deficiency abrogates recombinant Osteoactivin induced upregulation of Osterix and Col1α1, but not Runx2 in osteoblasts. (**A-C**) qPCR analysis of WT and CD44KO osteoblasts either left untreated (control; CTL) or treated with recombinant Osteoactivin (50 ng/mL) for 24 hours before termination. Briefly, osteoblasts were plated in 6 well plates at a seeding density of 200K cells per well and serum starved overnight. The next day, cells were either left untreated or treated with recombinant Osteoactivinfor 24 hours. Following treatment, RNA was isolated followed by qPCR analysis of osteoblast related genes Col1α1 (**A**), Osterix (**B**), or Runx2 (**C**) revealed that recombinant Osteoactivin upregulates these genes. This upregulation seems to be mediated through CD44 for Col1α1 and Osterix but not Runx2. The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean ± SEM. **=p<0.01 ***=p<0.001 compared to WT and untreated controls.

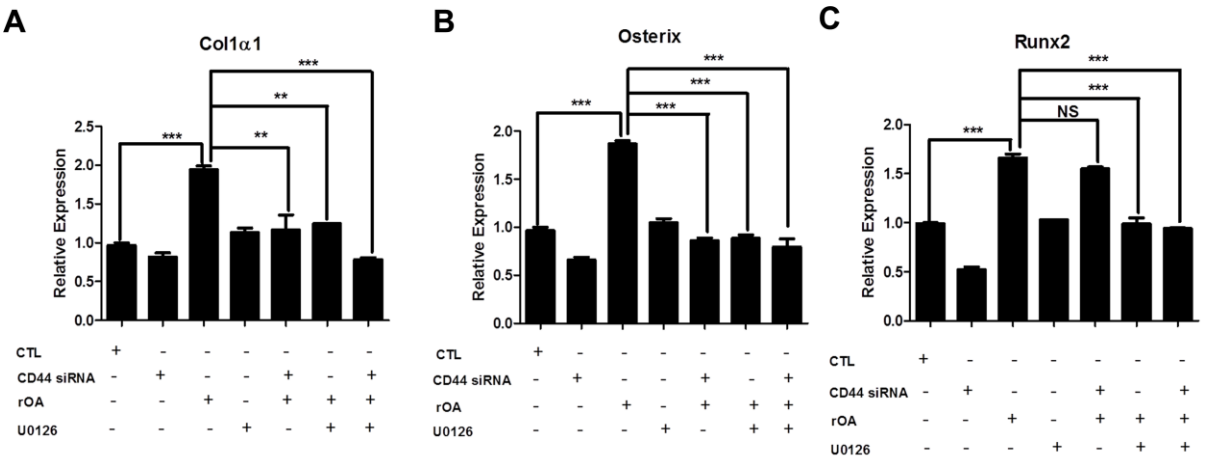


Figure 4.27: Recombinant Osteoactivin stimulates Col1α1 and Osterix through the CD44-ERK signaling axis. (**A-C**) qPCR analysis of MC3T3-E1 cells transfected with either scramble (CTL) or CD44 siRNA and either left untreated or treated with recombinant Osteoactivin (50ng/ml) or the ERK inhibitor U0126 (20μM) alone or in combination. Recombinant Osteoactivin upregulated osteoblast specific genes Col1α1 (**A**), Osterix (**B**), and Runx2 (**C**), however, inhibition of the ERK pathway abrogated the upregulation of all three genes. Interestingly, knockdown of CD44 only affected Osteoactivin induced upregulation of Col1α1 and Osterix, but not Runx2. Data presented in all graphs represent Mean ± SEM. **=p<0.01 ***=p<0.001.

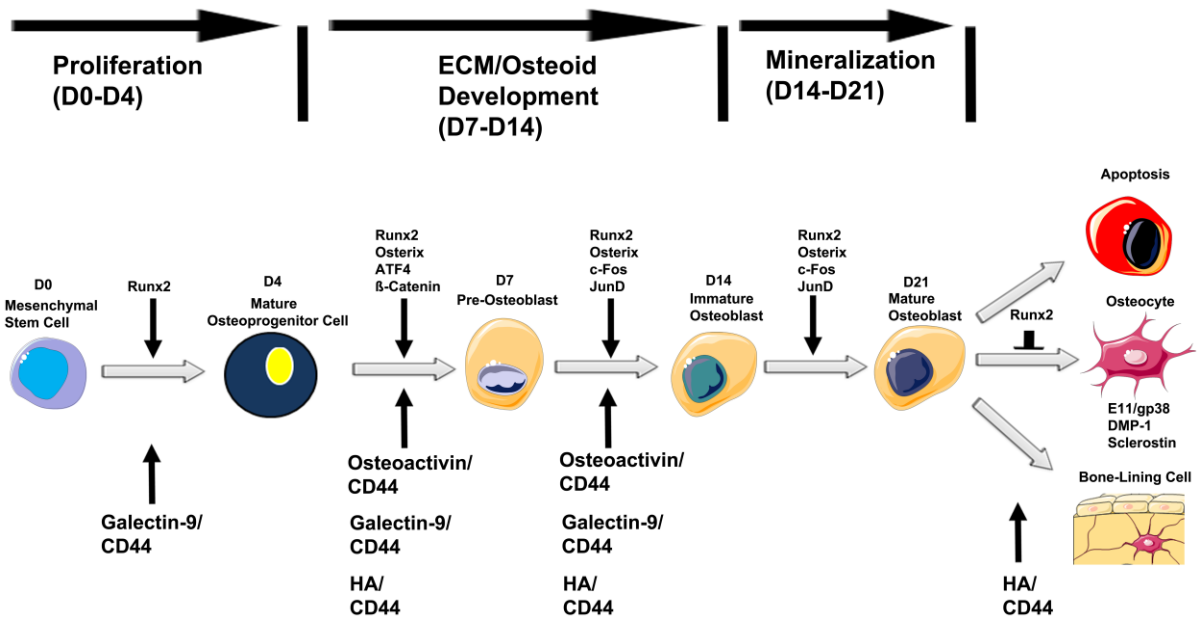


Figure 4.28: Schematic diagram depicting the stage at which OA-CD44 interaction stimulates osteoblast differentiation. Osteoactivin was shown to stimulate osteoblast differentiation and function. Our results indicate that during matrix deposition and extracellular matrix (ECM)/osteoid development Osteoactivin-CD44 interaction had its most potent effect. Interestingly, similar results at this stage have also been shown with other ligands of CD44 including Galectin-9 and Hyaluronan (HA).

4.11 Osteoactivin co-localizes within the Lipid Raft

Previous literature has shown that upon stimulation of CD44, clustering of CD44 with other receptors within lipid raft complexes can occur [450-452]. In this study, we wanted to determine if Osteoactivin has an association with cholesterol rich lipid raft micro domains. In order to determine if Osteoactivin co-localized within lipid raft micro domains, we used confocal immunoflourescent analysis of Osteoactivin and cholera-toxin B (CTX-B). CTX-B has binds to the GM1 ganglioside which has previously been shown to act as a resident raft marker [453, 454]. Immunoflourescent analysis revealed co-localization of Osteoactivin and CTX-B along the cell membrane (Figure 4.29).

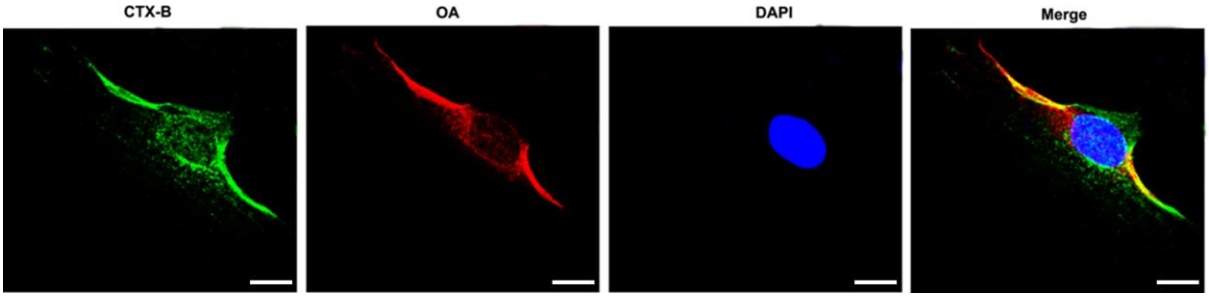


Figure 4.29: Osteoactivin is associated with cholesterol-rich lipid raft domains in osteoblasts. Immunoflourescent analysis of MC3T3-E1 cells probed for *Cholera* toxin-B (CTX-B; green) and Osteoactivin (OA; red). Briefly, cells were plated in chamber slides, fixed, and probed with an anti-Osteoactivin antibody. The next day, cells were treated with Alexa-flour 488 labeled CTX-B and secondary Alexa flour-568 antibody. Notice that OA co-localizes with CTX-B along the cell membrane. Scale bars: 10µm

4.12 Osteoactivin Treatment Stimulates Clustering of CD44 and Caveolin-1 in Lipid Rafts

Next, we wanted to determine if Osteoactivin treatment stimulated clustering of CD44 into lipid rafts. In order to determine if Osteoactivin induced clustering of CD44 into lipid rafts, we isolated soluble (SF) and lipid raft (RF) fractions by ultracentrifugation untreated and treated with either H3 or recombinant Osteoactivin (OA) and subjected them to immunoprecipitation and Western blot (Figure 4.30). The Hermes class 3 (H3) is a member of a class of adhesion molecules found to bind to CD44 during lymphocyte homing [455]. Cells treated with IgG (negative control) showed no enhancement of CD44 in lipid rafts; however, cells treated with H3 (positive control) showed an increase of CD44 compared to IgG control. Interestingly, recombinant Osteoactivin treatment increased the level of CD44 in lipid raft fractions compared to control.

In order to further elaborate on CD44 clustering induced by Osteoactivin stimulation, we treated cells with recombinant Osteoactivin (50ng/mL) and observed for co-localization of CD44 and lipid raft marker Caveolin-1 by confocal microscopy. Upon Osteoactivin stimulation, we observed co-localization of CD44 and Caveolin-1 along the membrane (Figure 4.31). These data indicate that Osteoactivin treatment results in the co-localization of CD44 into Cavolin-1 rich lipid rafts.

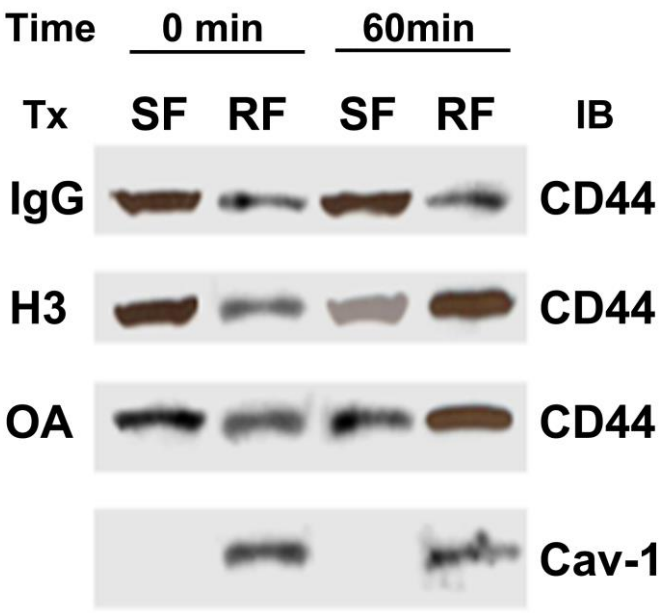


Figure 4.30: Recombinant Osteoactivin treatment induces CD44 to co-localize in lipid raft microdomains in osteoblasts. Western blot analysis of protein cell lysates from soluble (SF) and lipid raft (RF) fractions and treated with either IgG (negative control), H3 (positive control), or recombinant Osteoactivin (OA; 50ng/mL). Briefly, cells were plated in 6 well plates at a seeding density of 200K cells per well and serum starved overnight. The next day cells were either left untreated or treated with either IgG, H3, or OA for 60 minutes. Cell membrane preparations were separated by fractionation using an OptiPrep gradient by ultracentrifugation. Protein cell lysates were collected and analyzed by Western blot. Membranes were probed for an antibody against CD44 antibody. Anti-Caveolin-1 (Cav-1) antibodywas used to detect fractions positive for lipid rafts.

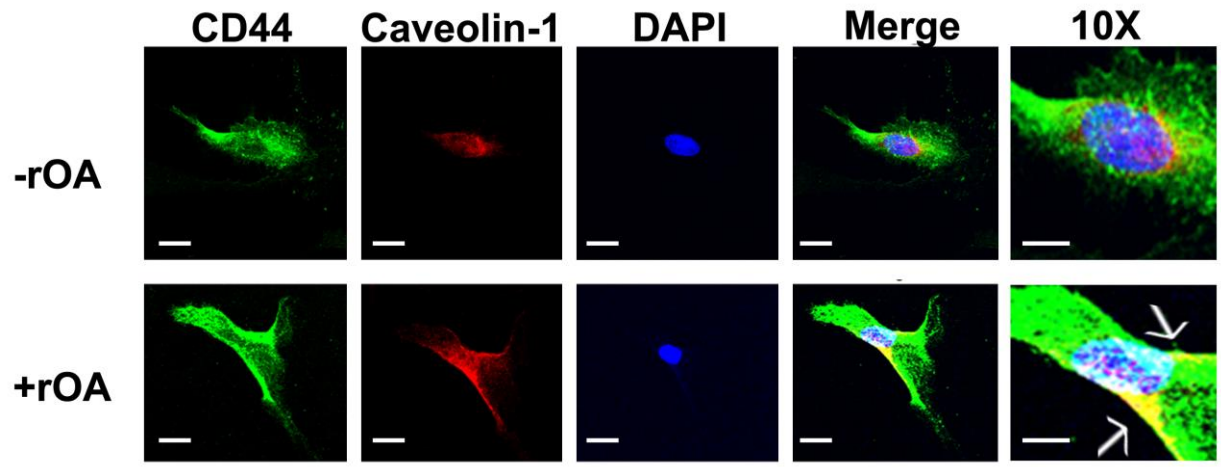


Figure 4.31: Recombinant Osteoactivin treatment induces co-localization of CD44 and Caveolin-1 in osteoblasts. Immunoflourescent analysis of MC3T3-E1 cells treated or untreated with recombinant Osteoactivin (rOA; 50ng/mL) and stained for CD44 and Caveolin-1. Briefly, cells were plated in chamber slides and serum starved overnight. The next day, cells were either left untreated or treated with rOA for 60 minutes at 37^0C. Cells were then fixed and stained with antibodies against CD44 and caveolin-1. The next day cells were treated with secondary Alexa-flour 488 and 568 antibodies. Scale bars: 10µm and 1µm (far right).

4.13 *Disruption of Lipid Raft Prevents CD44 and Caveolin-1 Clustering and Inhibits Osteoactivin Mediated ERK Phosphorylation in Osteoblasts*

In order to determine the role of the lipid raft in Osteoactivin/CD44 signaling, we used lipid raft inhibiting agents methyl-β-cyclodextrin (MβCD) and nystatin (Nyst.). Nystatin and MβCD have previously been reported to disrupt lipid rafts through the depletion of cholesterol [456-458]. First, we treated osteoblasts with recombinant Osteoactivin with or without MβCD to determine if disruption of the lipid raft would affect Osteoactivin mediated clustering of CD44 into lipid rafts. Recombinant Osteoactivin treatment resulted in the clustering of CD44 and Caveolin-1 by immunoprecipitation and Western blot analysis; however, when cells were treated with MβCD and Osteoactivin together, there was a reduction in CD44 and Caveolin-1 clustering compared to Osteoactivin treated alone (Figure 4.32).

Next, we wanted to determine if disruption of lipid raft affected Osteoactivin mediated signaling in osteoblasts. We treated cells with both recombinant Osteoactivin or nystatin alone or in combination to determine ERK activation by Western blot. Recombinant Osteoactivin, as previously shown in Figure 4.22, resulted in a dramatic increase in phosphorylated ERK. Interestingly, recombinant Osteoactivin when added in combination with nystatin resulted in a reduction in the level of phosphorylated ERK (Figure 4.33). In summary, these results reveal that disruption of the lipid raft results in the reduction of CD44/Caveolin-1 clustering and an inhibition of Osteoactivin induced ERK signaling. These results indicate the importance of CD44 and the lipid raft in Osteoactivin mediated signaling *in vitro*. Further evidence on the role of the lipid raft in Osteoactivin mediated osteoblast differentiation and function is warranted.

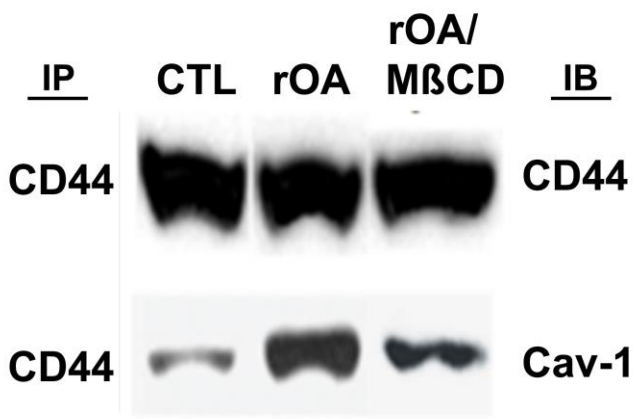


Figure 4.32: Lipid raft disruption prevents CD44 and Caveolin-1 clustering in osteoblasts. Western blot analysis of MC3T3-E1 cells either left untreated (control; CTL), treated with recombinant Osteoactivin (rOA; 50ng/mL), or treated with recombinant Osteoactivin and the lipid raft disrupting agent methyl-β cyclodextran (MβCD; 1mM). Briefly, MC3T3-E1 cells were plated in 6 well plates at a seeding density of 200K cells per well and serum starved overnight. The next day, the cells either remained untreated or treated with rOA for 60 minutes @ 37^0C. For disruption of the lipid raft, MβCD was added to cells for 60 minutes prior to the addition of rOA. Protein cell lysates were collected and immunoprecipitated with an anti-CD44 antibody. Immunoprecipitated proteins were analyzed by Western blot and membranes were probed for Caveolin-1 (Cav-1) and CD44. Notice the expression of Cav-1 in the rOA treated sample which is reduced in both rOA and MβCD treated samples.

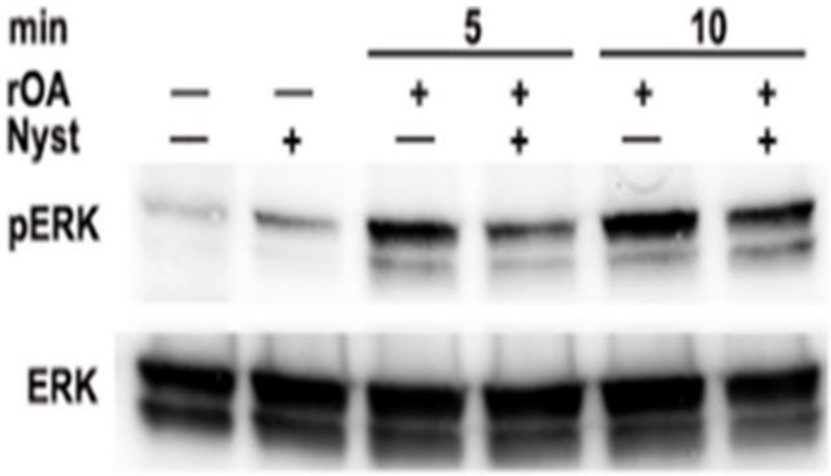


Figure 4.33: Lipid raft disruption prevents recombinant Osteoactivin-mediated increase in ERK signaling. Western blot analysis of MC3T3-E1 cells either left untreated, treated recombinant Osteoactivin (rOA; 50ng/mL), or rOA with the lipid raft inhibitor nystatin (Nyst; 50µg/mL). Briefly, cells were plated in 6 well plates at a seeding density of 200K cells per well and serum starved overnight. The next day, the cells either remained untreated or treated with rOA for different time points and incubated @ 37^0C. For disruption of the lipid raft, nystatin was added to cells for 60 minutes prior to the addition of rOA. Protein cell lysates were collected and analyzed by Western blot. Membranes were probed with antibodies against the phosphorylated and total ERK. Notice the decrease in phosphoERK in the rOA lipid raft disrupted samples.

4.14 Recombinant Osteoactivin Stimulates Autophagy in Osteoblasts

Previous studies have shown that caveolin rich lipid rafts can undergo endocytosis and stimulate cellular signaling [459]. Furthermore, previous literature has shown that endocytosis of caveolin-rich lipid rafts stimulate downstream signaling and autophagy [460, 461]. Autophagy has become an emerging new field in skeletal biology. Previous literature has shown that autophagy is an important regulator of osteoblast mineralization and bone homeostasis [462-464]. Since Osteoactivin seems to play a role in caveolin-rich lipid rafts, we hypothesized that recombinant Osteoactivin treatment may stimulate autophagy in osteoblasts. Microtubule-associated protein light chain 3 (LC3) is one of the most common indicators used to detect autophagy or autophagic flux. LC3 has two forms: LC3-I which is a cytoplasmic protein, and LC3-II which is conjugated to phosphatidylethanolamine on autophagosomal membranes [465]. Conversion of LC3-I to LC3-II is a good indicator of autophagic flux as the amount of LC3-II is closely correlated with the number of autophagosomes and autophagsome formation [466, 467]. Recombinant Osteoactivin treatment in osteoblasts displayed an enhancement in the conversion of LC3-I to LC3-II by Western blot analysis in a time dependent manner (Figure 4.34). Future studies should further examine the role of Osteoactivin in osteoblast autophagy.

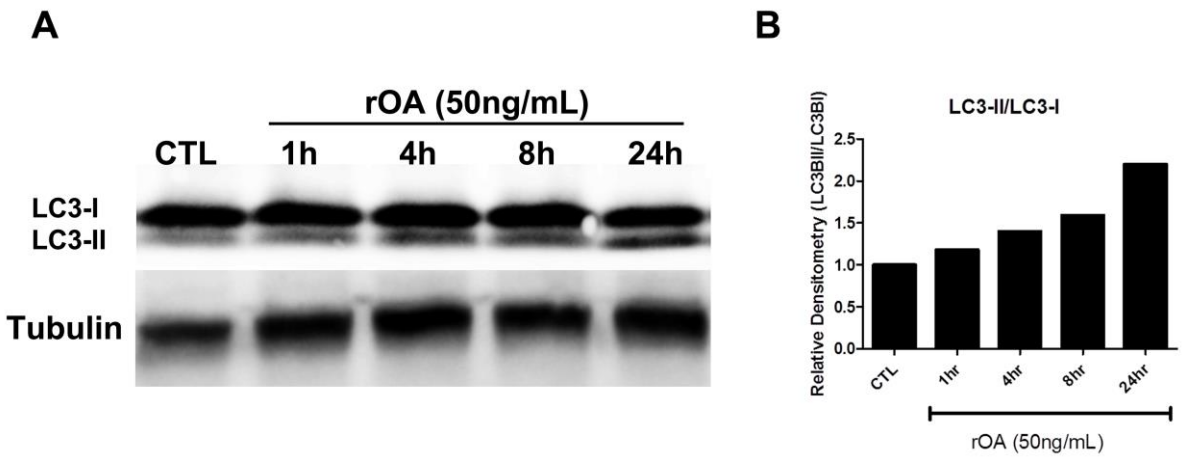


Figure 4.34: Recombinant Osteoactivin stimulates autophagy in osteoblasts. (**A-B**) Western blot (**A**) and Densitometry (**B**) analysis of MC3T3-E1 cells treated with recombinant Osteoactivin (50ng/mL) or left untreated and stained for LC3. MC3T3-E1 cells were plated in 6 well plates at a seeding density of 200K cells per well and low-serum (2% FBS) was added overnight. The next day, the cells either remained untreated or treated with rOA for different time points and incubated @ 37^0C. Protein cell lysates were collected and analyzed by Western blot. Membranes were probed with antibodies against LC3 and Tubulin (loading control). Notice the increase in LC3-II over time in the Osteoactivin treated samples.

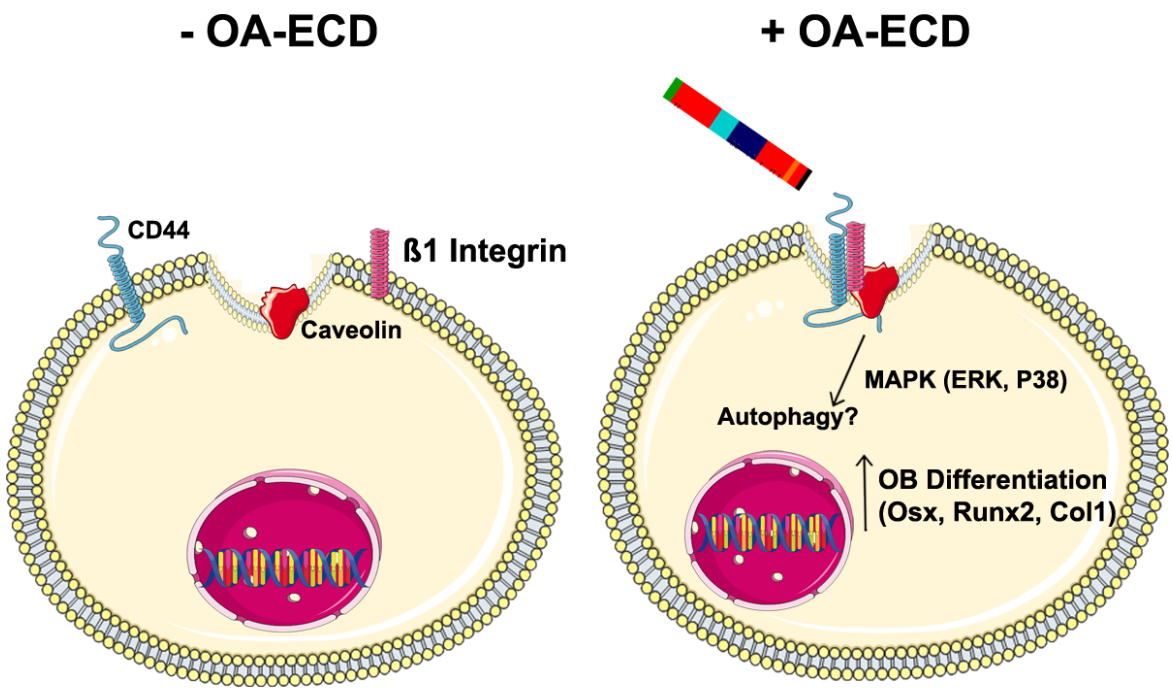


Figure 4.35: Schematic diagram of Osteoactivin signaling in osteoblasts. Osteoactivin extracellular domain (OA-ECD) has been shown to be shed from the membrane by ADAM10 in osteoblasts. Since the recombinant Osteoactivin mimics the shed extracellular domain, we believe that upon release of Osteoactivin from the membrane, Osteoactivin either acts in an autocrine or paracrine fashion and binds to CD44 where it stimulates a signling complex within the lipid raft. This complex induces intracellular signaling resulting in enhanced osteoblast differentiation. Interestingly, preliminary data suggests that autophagy may be involved in this process.

4.15 Osteoactivin Expression during Osteoclastogenesis

Previous literature has shown that Osteoactivin is expressed in osteoclasts [7-9]. The role of Osteoactivin in osteoclasts has been somewhat controversial. One study has shown that Osteoactivin is important for both osteoclast differentiation and function and that this process may be mediated through $\alpha_v\beta_3$ integrin [7, 9]. Recently our lab has reported that mutation in Osteoactivin in D2J mice results in enhanced osteoclast differentiation and an inhibition in resorption both *in vivo* and *ex vivo* suggesting that Osteoactivin plays a role in both osteoclast differentiation and function [8]. Although there are a few reports regarding the role of Osteoactivin in osteoclasts, the downstream signaling pathways related to Osteoactivin have not yet been determined. In this study, we examined the effect of recombinant Osteoactivin *in vitro* to determine its role in osteoclast differentiation and function.

In order to differentiate osteoclasts *in vitro*, inflammatory cytokines M-CSF and RANKL are required. Figure 4.36 represents a schematic diagram of the procedure that is necessary to differentiate osteoclasts. Certain cytokines such as NOTCH, γ-interferon and CX3CL1 have shown to have different effects on osteoclast differentiation based on when they are added in culture [468-471]. Recent work from our lab has shown recombinant Osteoactivin has different effects on osteoclast differentiation depending on when it is added in culture (unpublished results). In this study, recombinant Osteoactivin was added at day 3 and day 5 of osteoclast differentiation along with RANKL and M-CSF just prior to and during osteoclast commitment (Figure 36). In order to confirm that osteoclasts were able to be successfully differentiated, qPCR analysis of critical osteoclast transcription factors NFATc1 [472], c-Fos [199], and MITF [473] were

determined over the course of differentiation (Figure 4.37A). Interestingly, endogenous Osteoactivin levels were drastically higher compared to NFATc1, c-Fos, and MITF. Furthermore, the levels of Osteoactivin protein also increased during osteoclast differentiation as shown by ELISA (Figure 4.37B) and Western blot (Figure 4.37C). Interestingly, osteoclasts when treated with RANKL and M-SCF produce a significantly higher amount of Osteoactivin in the cell lysate compared to the conditioned media. This indicates that Osteoactivin that is produced by the osteoclasts remains primarily within the cell and only a fraction is released into the microenvironment.

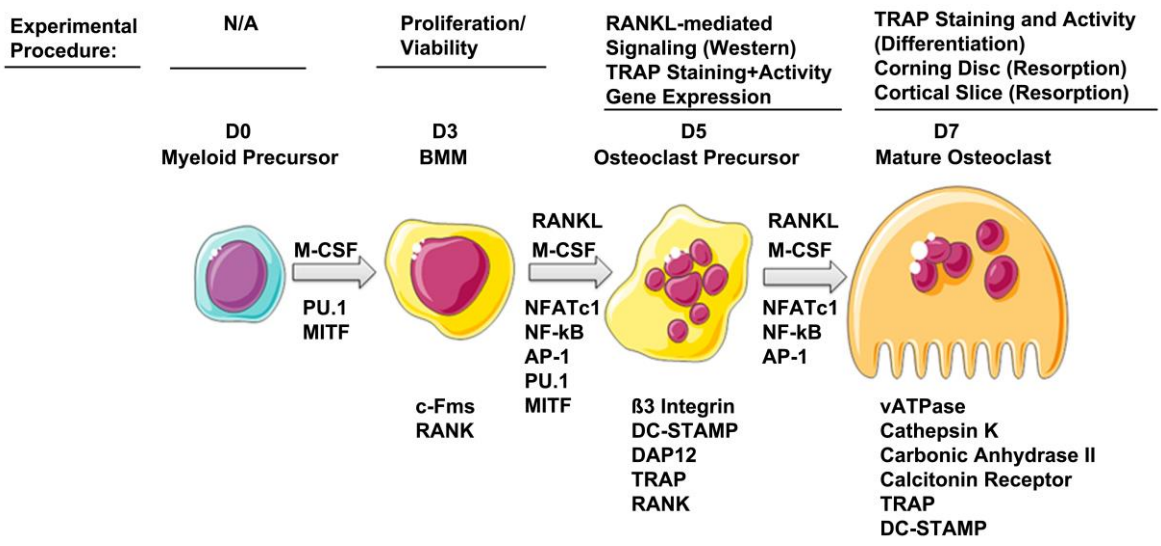


Figure 4.36: **Schematic diagram of osteoclast differentiation and the critical cytokines (above arrow), transcription factors (below arrow), and osteoclast specific markers (below cell) involved in the process.** Upon flushing of bone marrow, the beginning myeloid precursor starting at D0 begins to proliferate and differentiate with the help of the M-CSF and activated transcription factors PU.1 and MITF. After 72 hours bone-marrow macrophages (BMM) express high levels of c-Fms and RANK which stimulate downstream pathways critical for differentiation into osteoclast precursor cells upon M-CSF and RANKL treatment. After 48 hours of the first dose of RANKL and M-CSF treatment, a second dose of RANKL and M-CSF is given for another 48 hours in order for mature osteoclasts to develop. Recombinant Osteoactivin treatment is given each time RANKL and M-CSF is added.

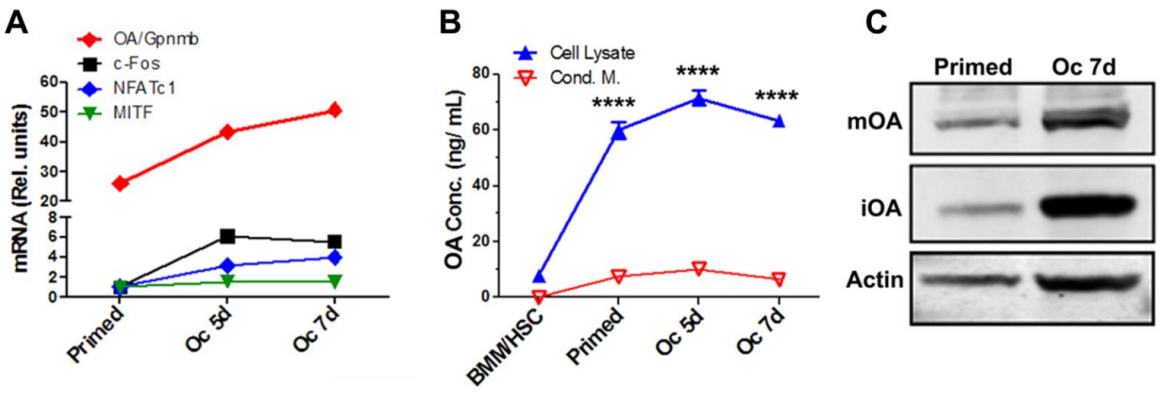


Figure 4.37: Osteoactivin (OA/Gpnmb) expression increases during osteoclast differentiation. Bone marrow derived from C57Blk6 (WT) mice was flushed and non-adherent cells (BMM) were cultured with 20 ng/mL M-CSF for 72 hours. Following the 72 hour period, primed BMM were given the first dose of RANKL (40 ng/mL) and M-CSF in order for primed cells to differentiate into pre-osteoclasts (Oc 5d). Forty-eight hours later pre-osteoclasts were given a second dose of RANKL and M-CSF for another 48 hours in order to differentiate into mature osteoclasts (Oc 7d). (**A**) qPCR analysis of Osteoactivin (OA/Gpnmb) mRNA expression during osteoclast differentiation. Notice OA/Gpnmb expression is high compared to known osteoclast transcription factors. (**B**) ELISA analysis of OA/Gpnmb expression during osteoclastogenesis. Notice the levels of Osteoactivin in the cell lysate increase during osteoclastogenesis, but levels in the conditioned media (Cond. M/CM) remain low. (**C**) Western blot analysis of both mature (mOA) and immature (iOA) Osteoactivin at early and late stage osteoclast differentiation. Data presented in all graphs represent Mean ± SEM. ********=p<0.0001 comparing cell lysate and conditioned media.

4.16 Osteoactivin Inhibits Osteoclast Differentiation in a Dose Dependent Manner

In order to determine the role of exogenous Osteoactivin treatment on osteoclast differentiation, we added recombinant Osteoactivin to BMM during osteoclast differentiation. Previous literature from our lab has shown that addition of recombinant Osteoactivin has no effect on BMM proliferation or survival (data not shown). Exogenous Osteoactivin treatment inhibited osteoclast differentiation determined by TRAP staining (Figure 4.38A) and activity (Figure 4.38B). Furthermore, recombinant Osteoactivin significantly inhibited osteoclast size and number (Figure 4.38C-F). Next, we wanted to determine the effect of recombinant Osteoactivin treatment on osteoclast related gene expression. Recombinant Osteoactivin treated BMM showed a significant reduction in osteoclast related gene expression of RANK, TRAP, DC-STAMP, NFATc1, OSCAR, and Calcitonin receptor both in a dose-dependent (Figure 4.39) and time-dependent manner (Figure 4.40). These results indicate that recombinant Osteoactivin treatment inhibits osteoclast differentiation *in vitro*.

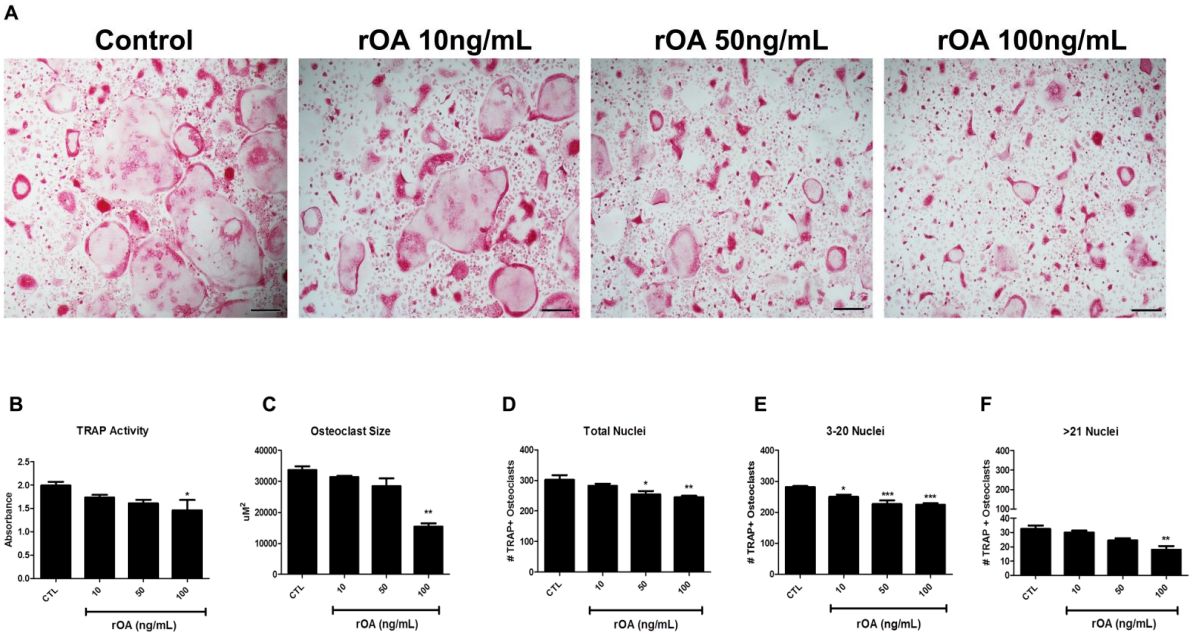


Figure 4.38: Recombinant Osteoactivin inhibits osteoclast differentiation *in vitro* in a dose dependent manner. BMM from WT cells were differentiated with M-CSF (20 ng/mL) and RANKL (40 ng/mL) along with increasing doses of recombinant Osteoactivin (rOA). (**A**) TRAP staining of mature osteoclasts untreated (Control) and treated with different doses of recombinant Osteoactivin. Notice as the concentration of Osteoactivin increases the TRAP Activity (**B**) and Osteoclast size (**C**) significantly decrease. (**D-F**) Osteoclast differential count showing total nuclei count (**D**), 3-20 nuceli (**E**), and >21 nuclei (**F**). The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean ± SEM. *****=p<0.05, ******=p<0.01, *******=p<0.001 comparing untreated controls (CTL) to treated samples. Scale bars: 500 µm (A, Top panel).

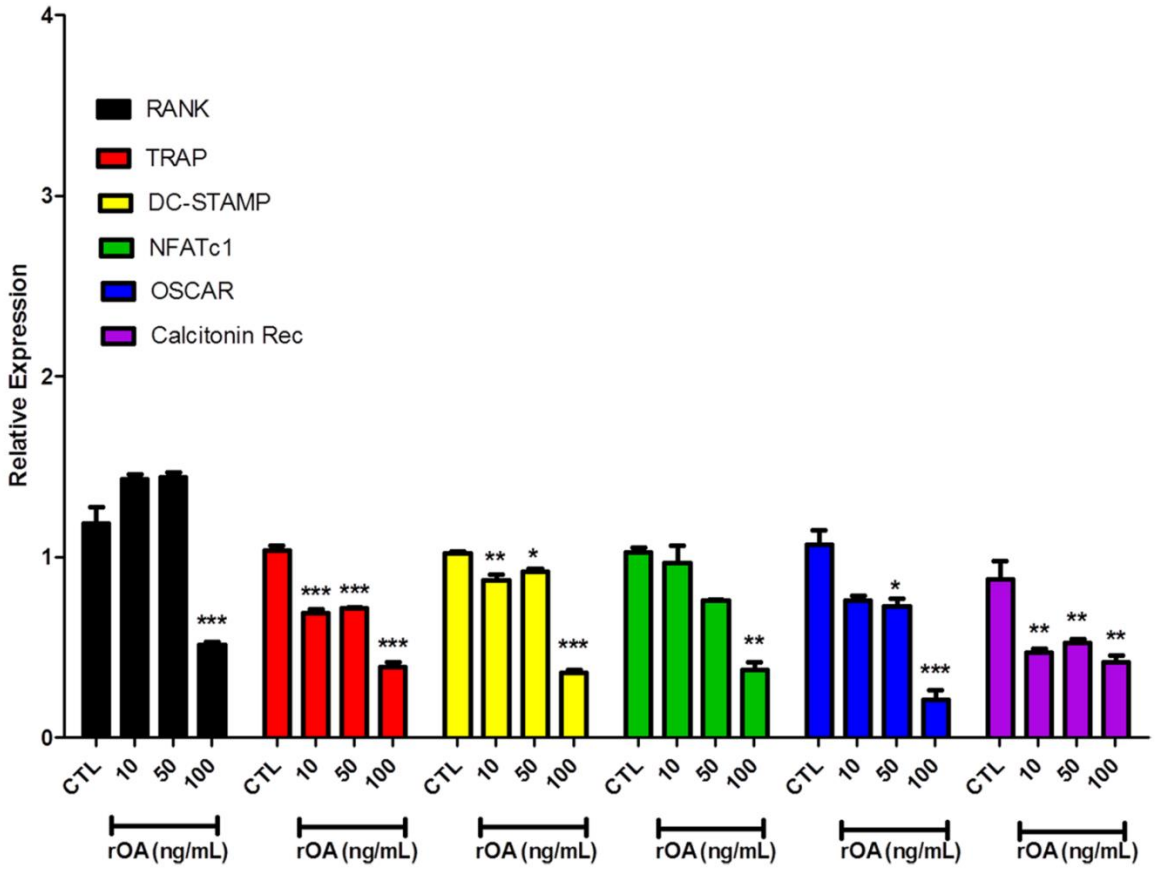


Figure 4.39: Recombinant Osteoactivin inhibits osteoclast related gene expression in a dose dependent manner. BMM from WT cells were differentiated with M-CSF (20ng/mL) and RANKL (40 ng/mL) along with increasing concentrations of recombinant Osteoactivin (rOA). Forty-eight hours later RNA was isolated from pre-osteoclasts (day 5) and converted into cDNA for qPCR analysis. Osteoactivin was shown to inhibit several important osteoclasts related markers including RANK, TRAP, DC-STAMP, NFATc1, OSCAR, and Calcitonin receptor in a dose dependent manner. The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean ± SEM. *****=p<0.05, ******=p<0.01, *******=p<0.001 comparing untreated control to treated samples.

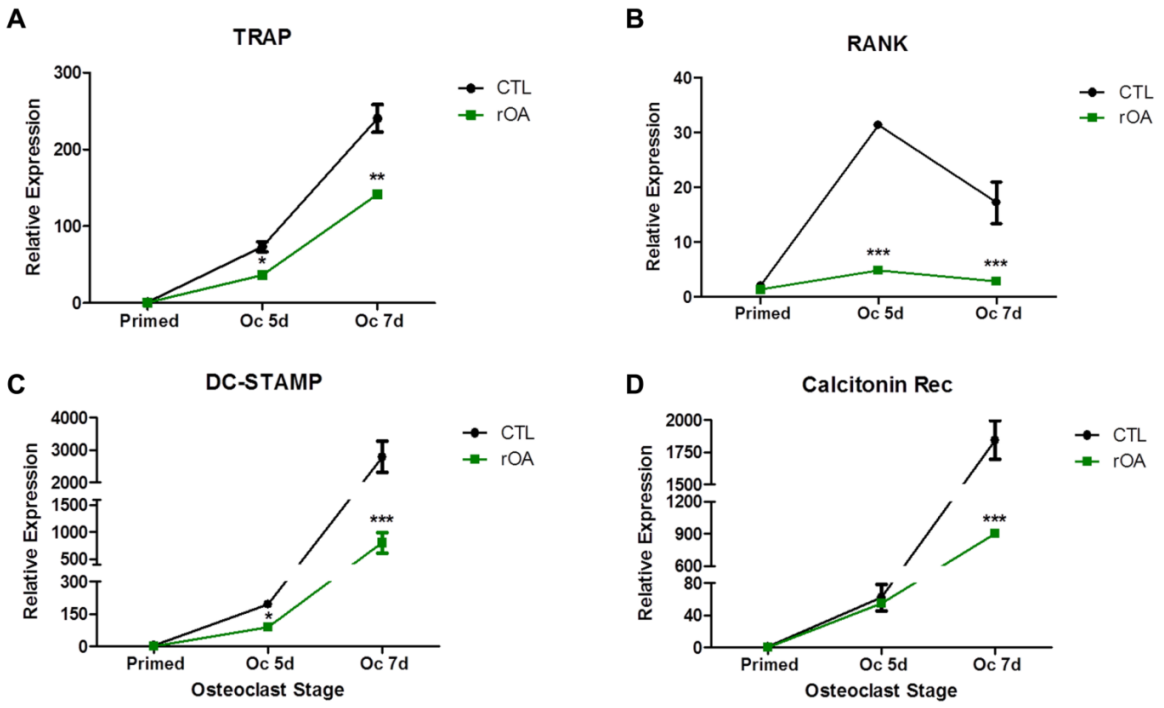


Figure 4.40: Recombinant Osteoactivin inhibits osteoclast gene expression during osteoclast differentiation. BMM from WT cells were differentiated with M-CSF (20 ng/mL) and RANKL (40 ng/mL) along with rOA (100 ng/mL). RNA was isolated during different stages of osteoclast differentiation in untreated (control;CTL) and treated (rOA) cells. (**A-D**) qPCR analysis of osteoclast related genes (**A**) TRAP, (**B**) RANK, (**C**) DC-STAMP, and (**D**) Calcitonin Receptor in untreated and rOA treated osteoclasts. Notice recombinant Osteoactivin treated samples significantly inhibit osteoclast related genes during differentiation which was highest during late stage differentiation. The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean ± SEM. ***=$p<0.05$, ****=$p<0.001$ comparing untreated control samples (CTL) to rOA treated samples.

4.17 The Effect of Recombinant Osteoactivin on RANKL Mediated Signaling During Osteoclastogenesis

The importance of the RANKL pathway including downstream signaling mediators and transcription factors has been previously described [164, 474, 475]. In order to determine the role of Osteoactivin during RANKL mediated signaling in osteoclasts, we pre-treated osteoclasts with recombinant Osteoactivin followed by RANKL treatment in a time-dependent manner and analyzed downstream mediators by Western blot analysis. Recombinant Osteoactivin treatment seemed to have no effect on either phosphoPLCγ2 (Figure 4.42A-B) or phosphoIκβ (Figure 4.43A-B) signaling. Further analysis on the members of the MAPK pathway revealed that recombinant Osteoactivin had no effect on either phosphoJNK (Figure 4.44AandD) or phosphoP38 (Figure 4.44CandF); however, treatment with recombinant Osteoactivin significantly reduced the levels of phosphorylatedERK compared to untreated controls (Figure 4.44BandE). Interestingly, AKT phosphorylation was also downregulated with the addition of recombinant Osteoactivin (Figure 4.45A-B). These results indicate that Osteoactivin inhibits RANKL mediated stimulation of the ERK and AKT pathways in osteoclasts.

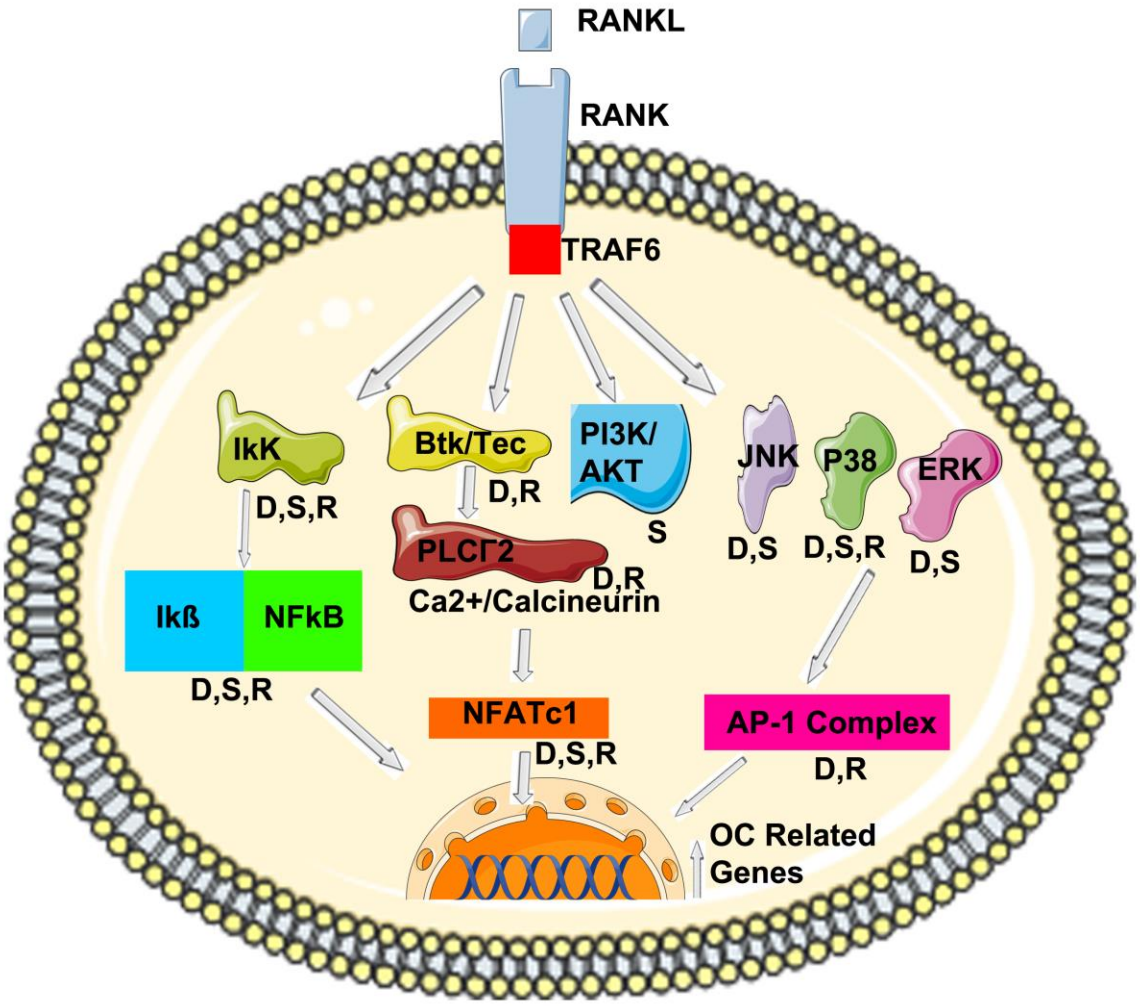


Figure 4.41: Schematic diagram of RANKL-mediated signaling pathways during osteoclastogenesis. RANKL/RANK stimulates several pathways critical for osteoclast differentiation. These include the NFκB, PLCγ, PI3K/AKT, and MAPK (JNK, P38, ERK) pathways. Upon RANKL and RANK binding, TRAF6 activates several mediators including IkK, Btk/Tec, PI3K, and Ras/MEK that regulate a variety of downstream transcription factors including NFκB, NFATc1, and the AP-1 complex. These transcription factors translocate to the nucleus and regulate several osteoclast related genes.

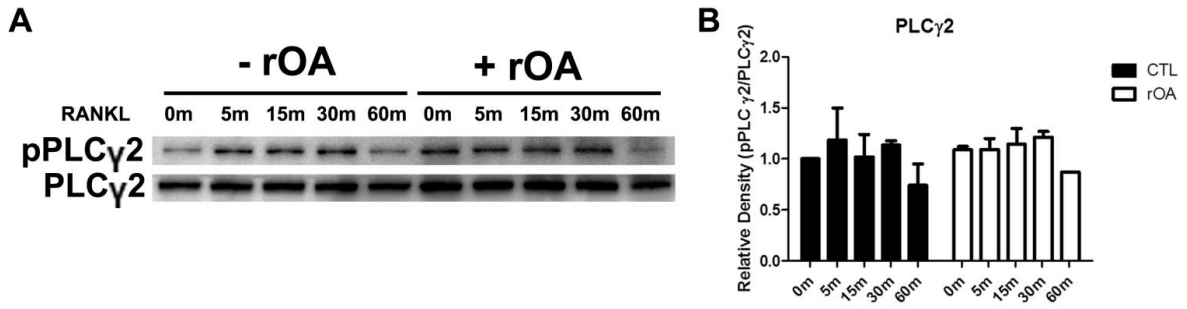


Figure 4.42: Recombinant Osteoactivin has no effect on RANKL mediated PLC$_\gamma$2 signaling in osteoclasts. BMM from WT cells were treated with M-CSF (20 ng/mL) and RANKL (40 ng/mL) for 48 hours with or without rOA (100 ng/mL). After the 48 hour period pre-osteoclasts were serum starved for 4 hours prior to RANKL (50 ng/mL) stimulation for the indicated time points. Total cell lysates were collected for Western blot analysis and probed with anti-phospho PLC$_\gamma$2 and anti-PLC$_\gamma$2 (**A**). Densitometric analysis (**B**) revealed no significant difference in untreated and treated samples. The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean ± SEM.

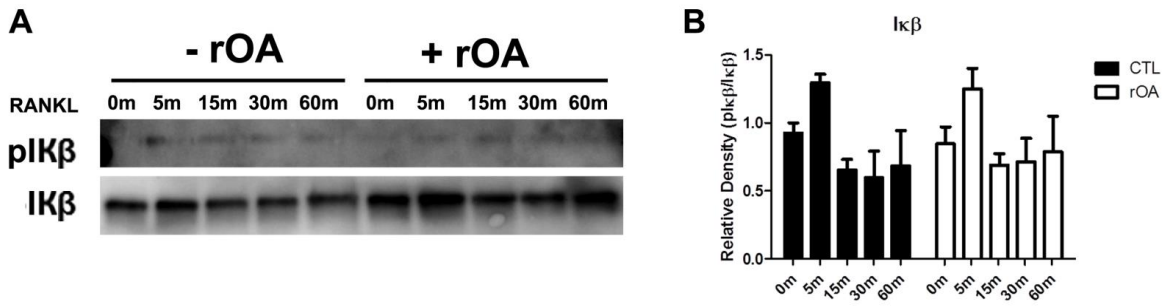


Figure 4.43: Recombinant Osteoactivin has no effect on RANKL induced Iκβ signaling in osteoclasts. BMM from WT cells were treated with M-CSF (20 ng/mL) and RANKL (40 ng/mL) for 48 hours with or without rOA (100 ng/mL). After the 48 hour period, pre-osteoclasts were serum starved for 4 hours prior to RANKL (50 ng/mL) stimulation for the indicated time points. Total cell lysates were collected for Western blot analysis and probed with anti-phospho Iκβ and anti-Iκβ (**A**). Densitometric analysis (**B**) revealed no significant difference between untreated and treated samples. The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean ± SEM.

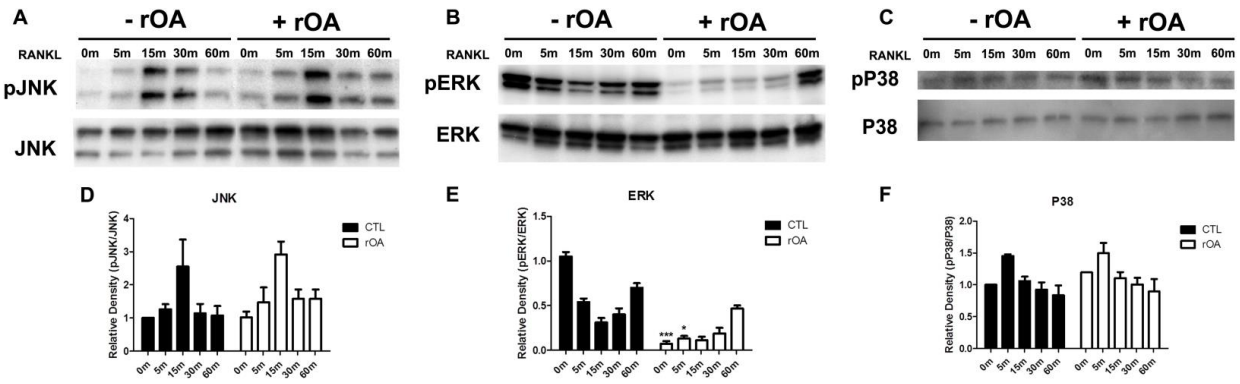


Figure 4.44: Recombinant Osteoactivin inhibits RANKL induced ERK activation in osteoclasts. BMM from WT cells were treated with M-CSF (20 ng/mL) and RANKL (40 ng/mL) for 48 hours with or without rOA (100ng /mL). After the 48 hour period, pre-osteoclasts were serum starved for 4 hours prior to RANKL stimulation for the indicated time points. Total cell lysates were collected for Western blot analysis and probed with anti-phospho JNK and anti-JNK (**A**), anti-phospho ERK and anti-ERK (**B**), and anti-phospho P38 and anti-P38 (**C**). Densitometric analysis (**D-F**) revealed no significant difference in untreated and rOA treated samples for JNK (**D**) and P38 (**F**); however, rOA treatment significantly down regulated levels of phosphoERK (**E**). The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean ± SEM. *****=p<0.05, *******=p<0.001 comparing untreated control and rOA treated samples.

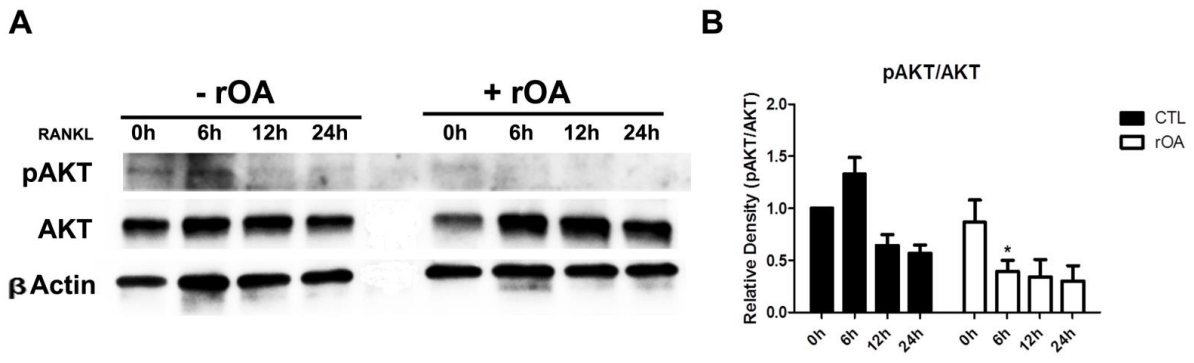


Figure 4.45: Recombinant Osteoactivin inhibits RANKL induced AKT signaling in osteoclasts. BMM from WT cells were treated with M-CSF (20 ng/mL) and RANKL (40 ng/mL) for 48 hours with or without rOA (100 ng/mL). After the 48 hour period, pre-osteoclasts (day 5) were serum starved for 4 hours prior to RANKL stimulation for the indicated time points. Total cell lysates were collected for Western blot analysis and probed with anti-phospho AKT and anti-AKT (**A**). Densitometric analysis (**B**) revealed a significant decrease in pAKT levels for recombinant Osteoactivin treated osteoclasts. The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean ± SEM. *****=$p<0.05$ comparing untreated control and rOA treated samples.

4.18 Osteoactivin Binds to CD44 in Osteoclasts

Previous work from our lab has shown that Osteoactivin binds to and interacts with CD44 on the osteoblast membrane (unpublished observations). In this study we wanted to determine if Osteoactivin interacts with CD44 on the osteoclast cell membrane. In order to determine if Osteoactivin colocalizes with CD44 on the osteoclast cell membrane, BMM isolated from wildtype mice were plated in chamber slides and differentiated into mature osteoclasts. Immunoflourescent analysis revealed that Osteoactivin and CD44 co-localize and interact on the plasma membrane of mature osteoclasts (Figure 4.46). Hyaluronan is the well-known and major ligand of CD44 [476]. In order to determine the interaction between Osteoactivin and CD44, high molecular weight hyaluronan was added to lysates and to cultures to show competitive binding between Osteoactivin and hyaluronan for CD44. Immunoprecipitation of CD44 and biotinylated recombinant Osteoactivin untreated and treated with high molecular weight hyaluronan was determined in order to assess Osteoactivin affinity for CD44. Immunoprecipitation analysis revealed that biotinylated recombinant Osteoactivin binds to CD44 and is displaced upon hyaluronan treatment (Figure 4.47). This indicates that Osteoactivin and hyaluronan may bind to CD44 at the same site.

Previous literature has shown that high molecular weight hyaluronan (1µg/mL) inhibits osteoclast differentiation through toll-like receptor 4 (TLR-4) *in vitro* [440]. In order to determine the interaction between hyaluronan, CD44, and Osteoactivin during osteoclastogenesis, we added both recombinant Osteoactivin and hyaluronan during osteoclast differentiation. Recombinant Osteoactivin and hyaluronan alone inhibited osteoclasts as expected. Hyaluronan and recombinant Osteoactivin added in

combination inhibited osteoclast differentiation; however, the effect was not synergistic (Figure 4.48). Furthermore, we added recombinant Osteoactivin and hyaluronan to WT and CD44KO cells. Recombinant Osteoactivin and HA treatment showed similar patterns in WT as previously described. Interestingly, addition of Osteoactivin to CD44 deficient cells had no effect on osteoclast differentiation compared to control; however, HA treatment alone inhibited osteoclasts, and HA in combination with recombinant Osteoactivin also inhibited osteoclasts. This indicates that Osteoactivin may inhibit osteoclasts through CD44 and that HA inhibits osteoclasts through TLR-4 not CD44 as previously described [440].

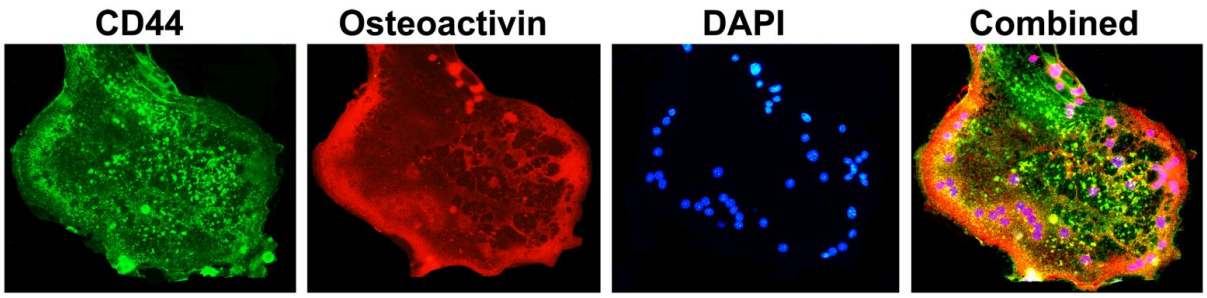


Figure 4.46: Osteoactivin co-localizes with CD44 in mature osteoclasts. BMM from WT cells were differentiated into mature osteoclasts with M-CSF (20ng/ml) and RANKL (50ng/mL) and terminated at day 7. Immunoflourescent staining of mature osteoclasts was used to visualize the expression of CD44 and Osteoactivin in multinucleated osteoclasts. Notice that CD44 and Osteoactvin seem to co-localize primarily along the cell membrane.

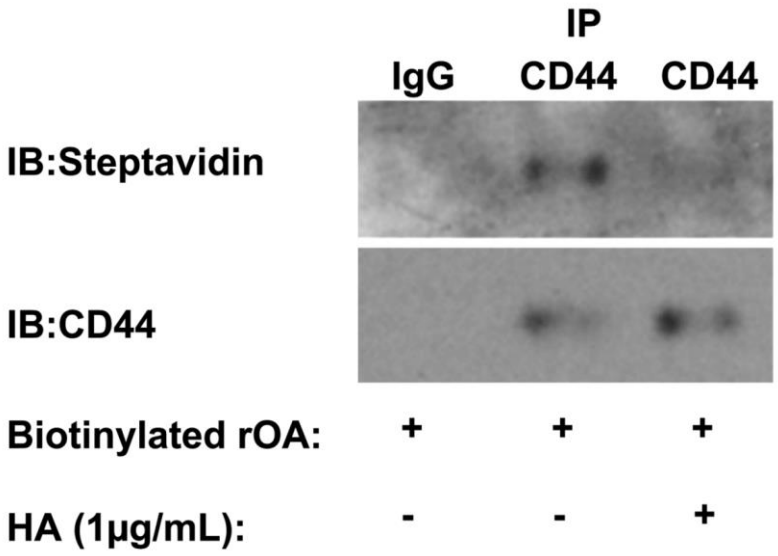


Figure 4.47: Osteoactivin binds to CD44 and is displaced upon hyaluronan treatment. BMM from WT cells were differentiated into pre-osteoclasts with M-CSF and RANKL for 48 hours. After the 48 hour period, cells were serum starved and treated with biotinylated recombinant Osteoactivin (rOA) alone or in combination with hyaluronan (HA). Protein cell lysates were collected and subjected to immunoprecipitation analysis. Cell lysates were incubated with IgG or CD44 antibodies for immunoprecipitation and subjected to Western blot analysis. Blots were probed with anti-streptavidin and anti-CD44. Notice that when CD44 is immunoprecipitated with rOA treatment alone a distinct band can be seen; however upon the addition of HA, the band disappears indicating HA and rOA compete for the same site of CD44.

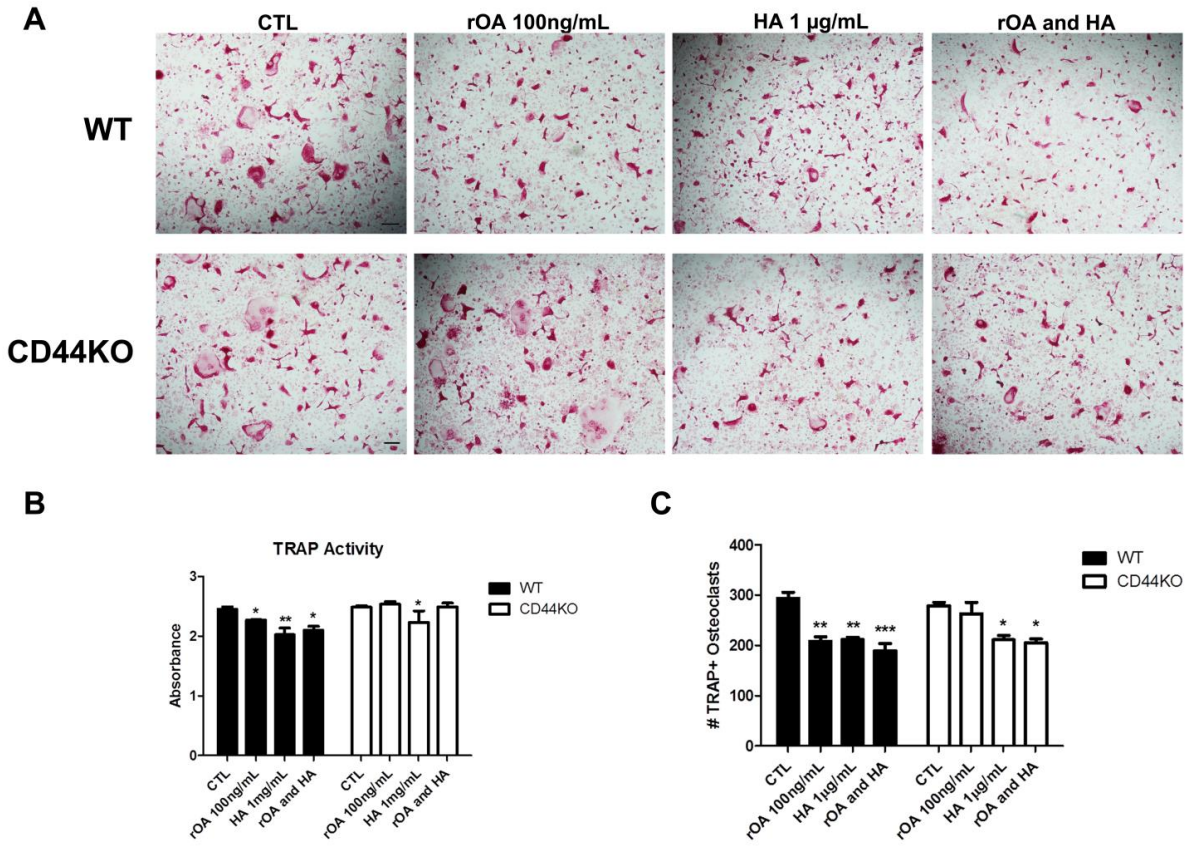


Figure 4.48: The inhibition of recombinant Osteoactivin is abrogated in CD44KO cells alone and in combination with hyaluronan. BMM from WT and CD44KO cells were differentiated with M-CSF (20ng/mL) and RANKL (50ng/mL) alone (CTL) or with rOA (100ng/mL) and hyaluronan (HA; 1µg/mL) alone, or in combination (rOA+HA) for 7 days to generate mature osteoclasts. (**A-C**) Mature osteoclasts were analyzed for TRAP staining (**A**), activity (**B**), and count (**C**) among samples to determine the effect of rOA and HA together. Notice that rOA and HA alone inhibit osteoclast differentiation; however in combination, an enhanced inhibitory response is not seen indicating a competition between the two ligands for the same receptor. Data presented in all graphs represent Mean ± SEM. ***=p<0.05, ****=p<0.001. Scale bars: 500 µm (Panel A) comparing untreated controls (CTL) to treated samples.

4.19 Osteoactvin Mediated Inhibition of Osteoclastogenesis is Abrogated in CD44 Deficient Osteoclasts

In order to further examine whether Osteoactivin inhibition of osteoclasts is CD44 dependent, we used CD44 deficient mice (CD44KO) for osteoclast culture (Figure 4.49). Recombinant Osteoactivin added to wild type (WT) cells showed a significant reduction in osteoclast differentiation by TRAP staining (Figure 4.50A), activity (Figure 4.50B), and count (Figure 4.50C); however, this effect was abrogated when CD44 deficient osteoclasts were treated with recombinant Osteoactivin. This indicates that CD44 is involved in the Osteoactivin-mediated inhibition of osteoclastogenesis. To elaborate upon this idea, we repeated this same experiment with osteoclasts grown on bovine cortical slices in order to determine if osteoclasts grown on a surface that mimics the bone extracellular matrix has different effects. The results showed similar responses to osteoclasts grown on plastic. Osteoactivin inhibited osteoclast differentiation in WT cells by TRAP staining (Figure 4.51A), activity (Figure 4.51B), and count (Figure 4.51C); however, this effect was abrogated in CD44 deficient cells. Next, we wanted to determine if CD44 was responsible for the Osteoactivin-mediated inhibition of osteoclast gene expression. Recombinant Osteoactivin added to WT BMM inhibited osteoclast related genes TRAP (Figure 4.52A), NFATc1 (Figure 4.52B), RANK (Figure 4.52C), DC-STAMP (Figure 4.52D), and calcitonin receptor (Figure 4.52E); however, when Osteoactivin was added to CD44 deficient cells, this effect was abrogated if not enhanced. This indicates that Osteoactivin-mediated inhibition of osteoclast related genes is through the CD44 receptor.

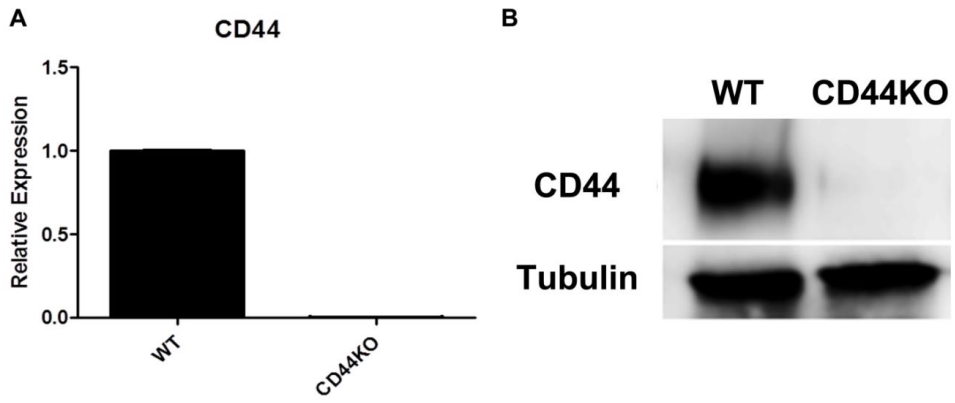


Figure 4.49: Confirmation of the absence of CD44 in CD44 deficient osteoclasts. BMM from WT and CD44 deficient cells (CD44KO) were differentiated with M-CSF (20ng/mL) and RANKL (40ng/mL) for 7 days to generate mature osteoclasts. The absence of CD44 was confirmed by both mRNA and protein using qPCR (**A**) and Western blot (**B**) analysis respectively. Notice that CD44 was undetected in the graph and in the blot.

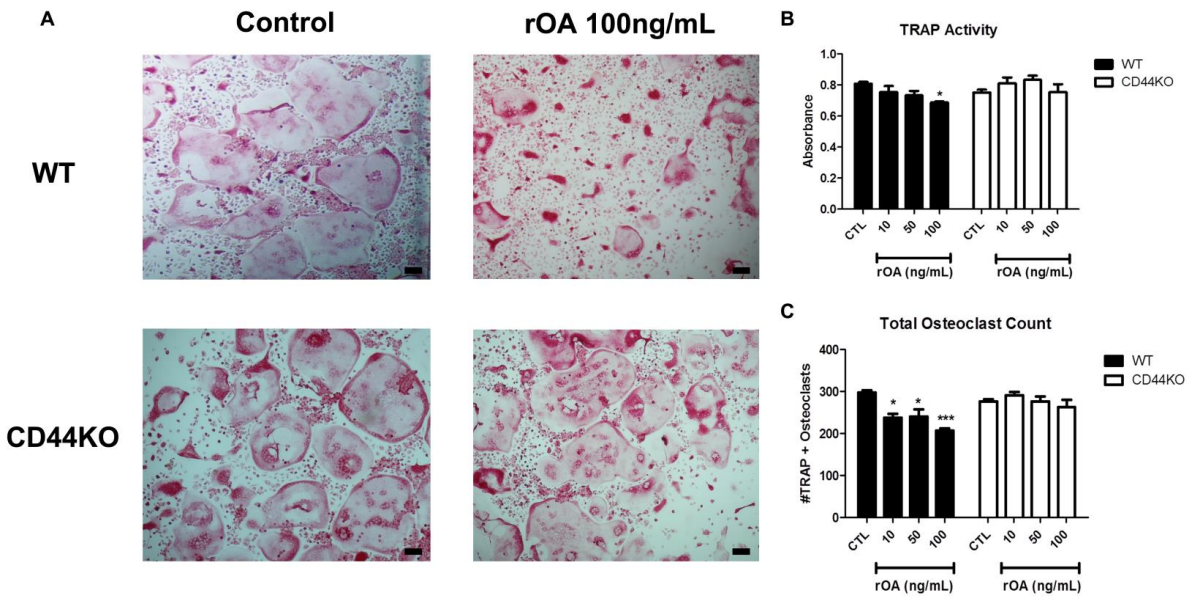


Figure 4.50: Osteoactivin mediated inhibition of osteoclast differentiation is abrogated in CD44 deficient cells cultured on plastic. BMM from WT and CD44KO cells were differentiated with M-CSF (20ng/mL) and RANKL (40ng/mL) alone (Control) or in combination with rOA for 7 days to generate mature osteoclasts. (**A**) Microscopic images of TRAP positive osteoclasts show an inhibition upon rOA treatment in WT samples; however, in CD44 deficient cells there is no effect upon rOA treatment. (**B-C**) Osteoclast parameters reveal an inhibition of osteoclast differentiation in rOA treated WT cells shown by (**B**) TRAP activity and osteoclast count (**C**). This effect is abrogated in the CD44KO samples. The experiment had 6-replicates and was repeated 3 times with similar results. Data presented in all graphs represent Mean ± SEM. *****=$p<0.05$, *******=$p<0.001$ comparing untreated controls (CTL) to treated samples. Scale bars: 100 µm (Panel A).

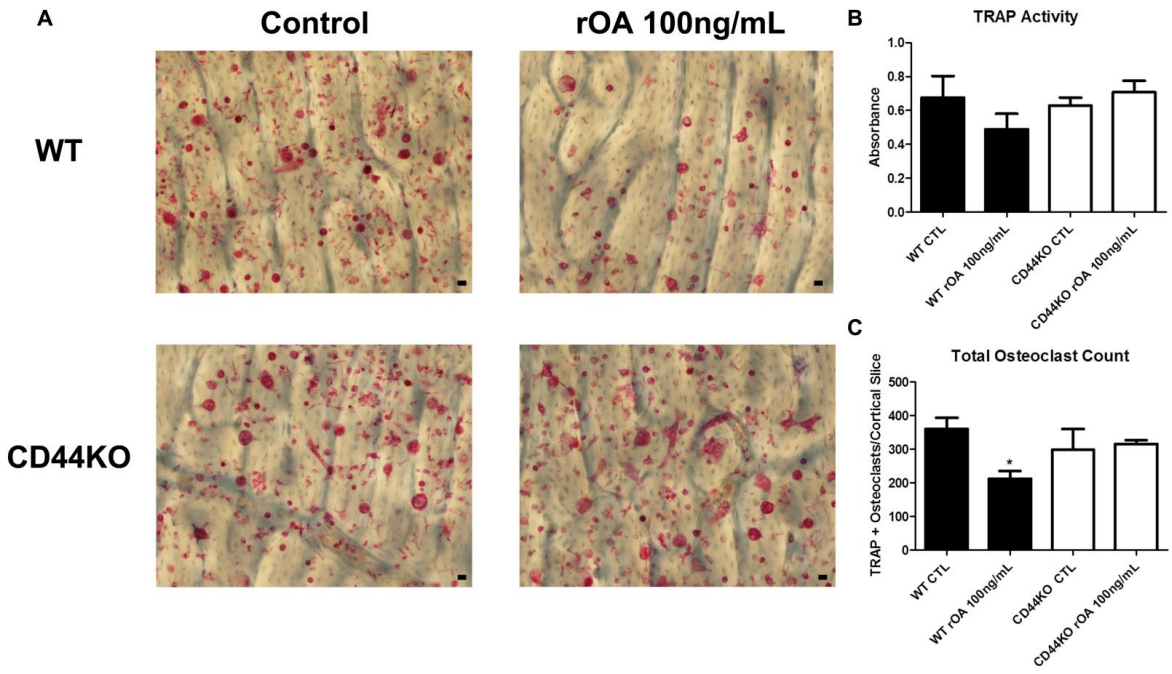


Figure 4.51: Osteoactivin mediated inhibition of osteoclast differentiation is abrogated in CD44 deficient cells cultured on cortical bone slices. BMM from WT and CD44KO cells were differentiated with M-CSF and RANKL alone (Control) or in combination with rOA for 7 days to generate mature osteoclasts. (**A**) Microscopic images of TRAP positive osteoclasts show an inhibition upon rOA treatment in WT samples; however, in CD44KO cells there is no effect upon rOA treatment. (**B-C**) Osteoclast parameters reveal an inhibition of osteoclast differentiation in rOA treated WT cells shown by (**B**) TRAP activity and osteoclast count (**C**). This effect is abrogated in the CD44KO samples. The experiment was run in triplicates and repeated 3 times with similar results. Data presented in all graphs represent Mean ± SEM. *****=$p<0.05$. Scale bars: 100 µm (Panel A).

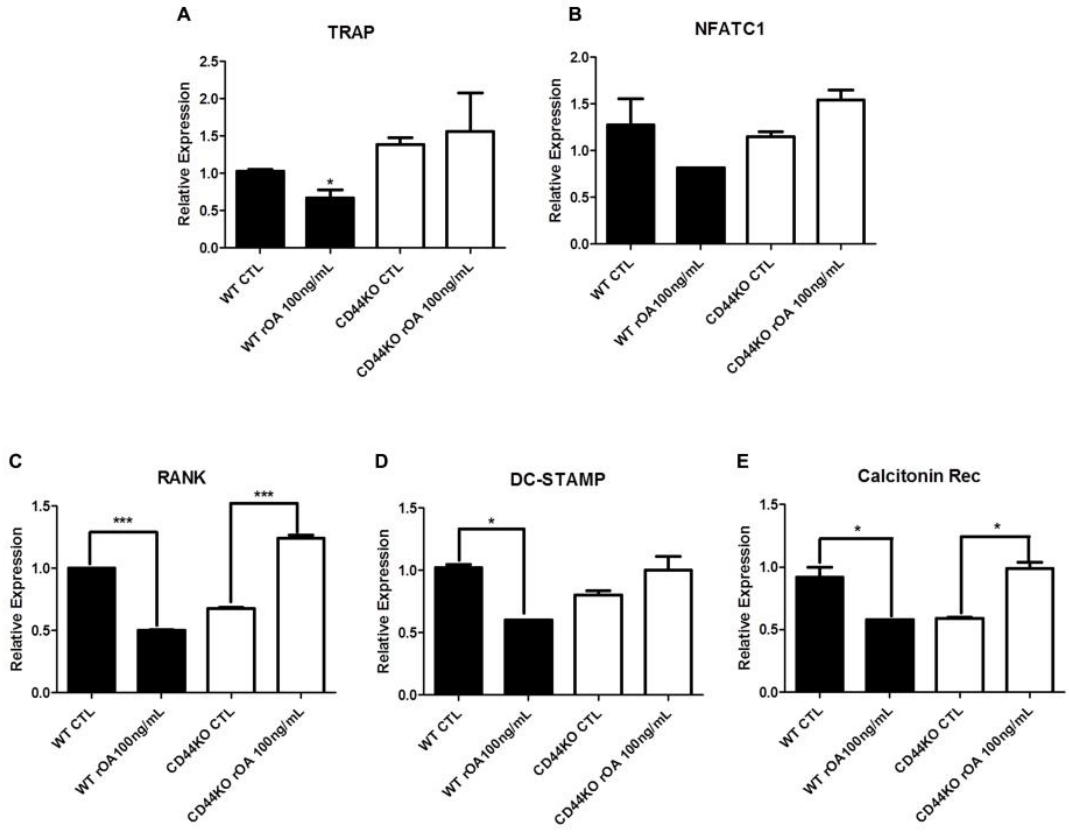


Figure 4.52: Osteoactivin mediated inhibition of osteoclast related gene expression is abrogated in CD44 deficient cells. BMM from WT and CD44KO cells were differentiated with M-CSF (20ng/mL) or RANKL (40ng/mL) alone (CTL) or in combination with rOA (100 ng/mL) for 48 hours to generate pre-osteoclasts. (**A-E**) qPCR analysis of osteoclasts from WT and CD44KO cells either treated or untreated with rOA to determine the relative expression of osteoclast related genes TRAP (**A**), NFATc1 (**B**), RANK (**C**), DC-STAMP (**D**), and Calcitonin Receptor (**E**). Notice upon treatment of rOA to WT cells osteoclast related genes are downregulated; however this effect is abrogated in CD44KO cells. The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean ± SEM. ***=p<0.05, ****=p<.001.

4.20 Osteoactivin Inhibition of ERK Phosphorylation is mediated through CD44

In order to determine the signaling pathway involved in Osteoactivin mediated osteoclast inhibition, we used WT and CD44 deficient mice and examined RANKL-mediated stimulation of osteoclast signaling in response to osteoactivin. Previous literature has shown that loss of CD44 has no effect on osteoclast differentiation *in vitro* [393]. Similarly, we have shown that loss of CD44 has no effect on RANKL-mediated osteoclast signaling *in vitro* (Figure 4.53). In Figure 4.44, we have shown that addition of recombinant Osteoactivin in RANKL stimulated osteoclasts inhibits ERK phosphorylation. Addition of Osteoactivin in WT cells inhibited ERK phosphorylation as expected. Interestingly, the addition of Osteoactivin to CD44 deficient osteoclasts resulted in restoration of ERK phosphorylation (Figure 4.54). This indicates that Osteoactivin inhibition of ERK phosphorylation is mediated through CD44.

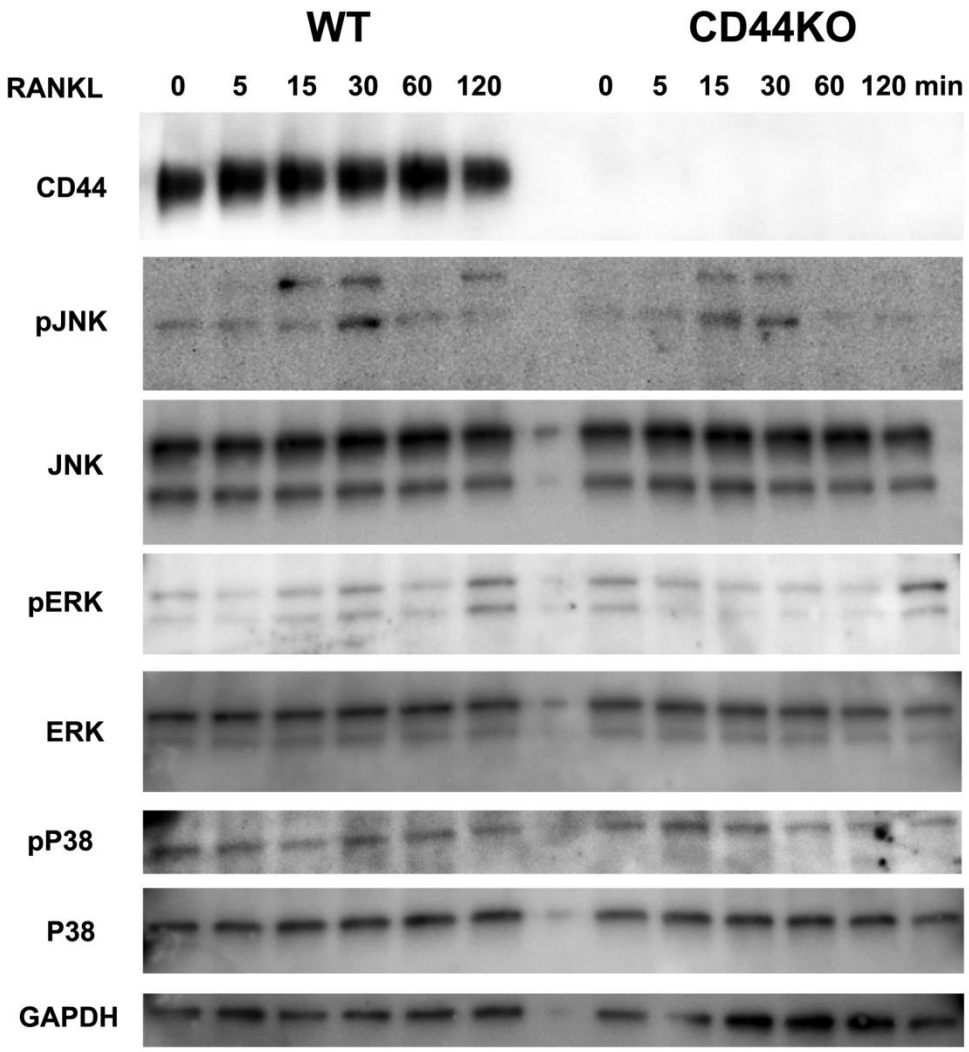


Figure 4.53: RANKL induced signaling is unaltered in CD44 deficient osteoclasts. BMM from WT and CD44KO cells were treated with M-CSF (20ng/mL) and RANKL (40ng/mL) for 48 hours in order to generate pre-osteoclasts. After the 48 hour period, cells were serum starved for 4 hours prior to the addition of RANKL (50 ng/mL) for the indicated time periods. Total protein cell lysates were collected and analyzed by Western blot. Blots were probed for antibodies of the MAPK family. Notice there does not seem to be a difference in endogenous RANKL signaling between WT and CD44KO cells.

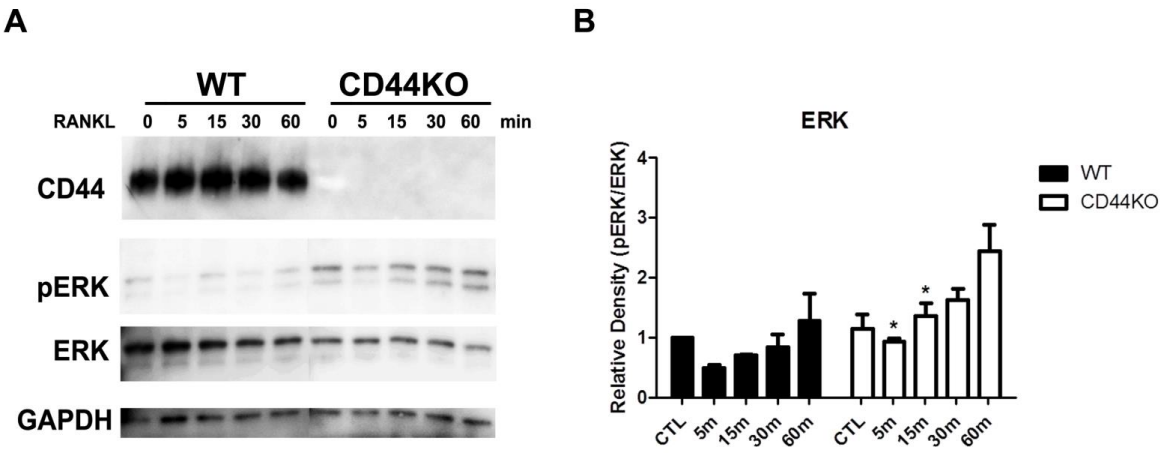


Figure 4.54: Osteoactivin mediated inhibition of ERK signaling is abrogated in CD44 deficient cells. BMM from WT and CD44KO cells were treated with M-CSF (20ng/mL) and RANKL (40ng/mL) along with rOA (100 ng/mL) for 48 hours in order to generate pre-osteoclasts. After the 48 hour period, cells were serum starved for 4 hours prior to the addition of RANKL (50 ng/mL) for the indicated time periods. Total protein cell lysates were collected and analyzed by Western blot (**A**). Blots were probed for antibodies anti-CD44, anti-phospho ERK, anti-ERK, and anti-GAPDH. (**B**) Densitometric analysis reveals a significant increase in ERK signaling upon rOA treatment in CD44 deficent cells compared to WT. The experiment was repeated 3 times with similar results. Data presented in all graphs represent Mean ± SEM. *****=$p<0.05$ comparing untreated and treated samples from WT and CD44KO.

4.21 The Effect of Recombinant Osteoactivin in WT and CD44 Deficient Cells in vivo

Previous studies have shown that addition of certain anti-inflammatory cytokines can inhibit osteoclast differentiation and migration in a calvaria osteolysis model [477-480]. Therefore, we wanted to examine the effects of recombinant Osteoactivin on osteoclast differentiation and recruitment *in vivo*. In WT cells, addition of RANKL significantly increased the number of TRAP positive osteoclasts as expected; however, addition of RANKL in combination with recombinant Osteoactivin abrogated the RANKL stimulation of osteoclast differentiation and recruitment by TRAP staining (Figure 4.55) and microCT (Figure 4.56). To further elucidate the mechanism involved in the Osteoactivin mediated inhibition of osteoclasts *in vivo*, we tested recombinant Osteoactivin using the same model in CD44 deficient mice. As expected, RANKL alone stimulated osteoclast differentiation and recruitment. Interestingly, RANKL treatment, in combination with Osteoactivin, also stimulated osteoclast differentiation and recruitment in the CD44 deficient mice. This indicates that Osteoactivin inhibits RANKL mediated osteoclast differentiation through the CD44 pathway *in vivo*.

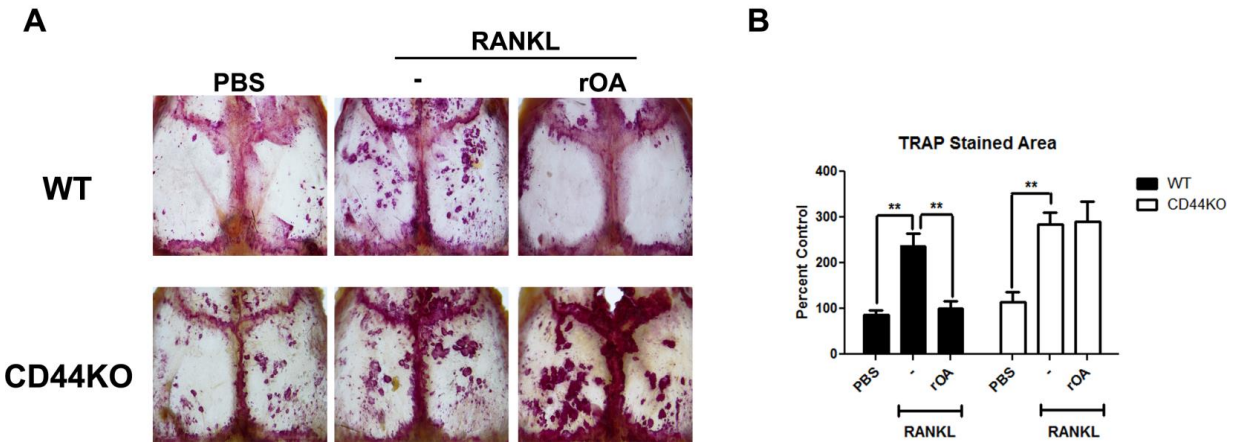


Figure 4.55: Recombinant Osteoactivin inhibits the differentiation and recruitment of TRAP positive osteoclasts *in vivo*. A collagen sponge coated with 30 µL of either PBS, RANKL (5 µg) alone, or RANKL and rOA (100 µg/mL) was placed on WT or CD44KO calvaria (n=5) for seven days. A week later calvaria were removed and TRAP stained as described in Materials and Methods. (**A**) Stereoscope images were taken of TRAP stained calvaria for each condition. Notice the increased number of osteoclasts in RANKL alone treatments which is inhibited in the rOA and RANKL combined treatment in WT cells. (**B**) TRAP stained area analysis reveals an increase in the overall TRAP stained area in RANKL treated WT calvaria which is reduced in rOA and RANKL combined treatments in WT. This effect seems to be abrogated in CD44KO calvaria as a reduction in the TRAP stained area is not observed in rOA and RANKL treatment compared to RANKL alone. Data presented in all graphs represent Mean ± SEM. **=$p<0.01$.

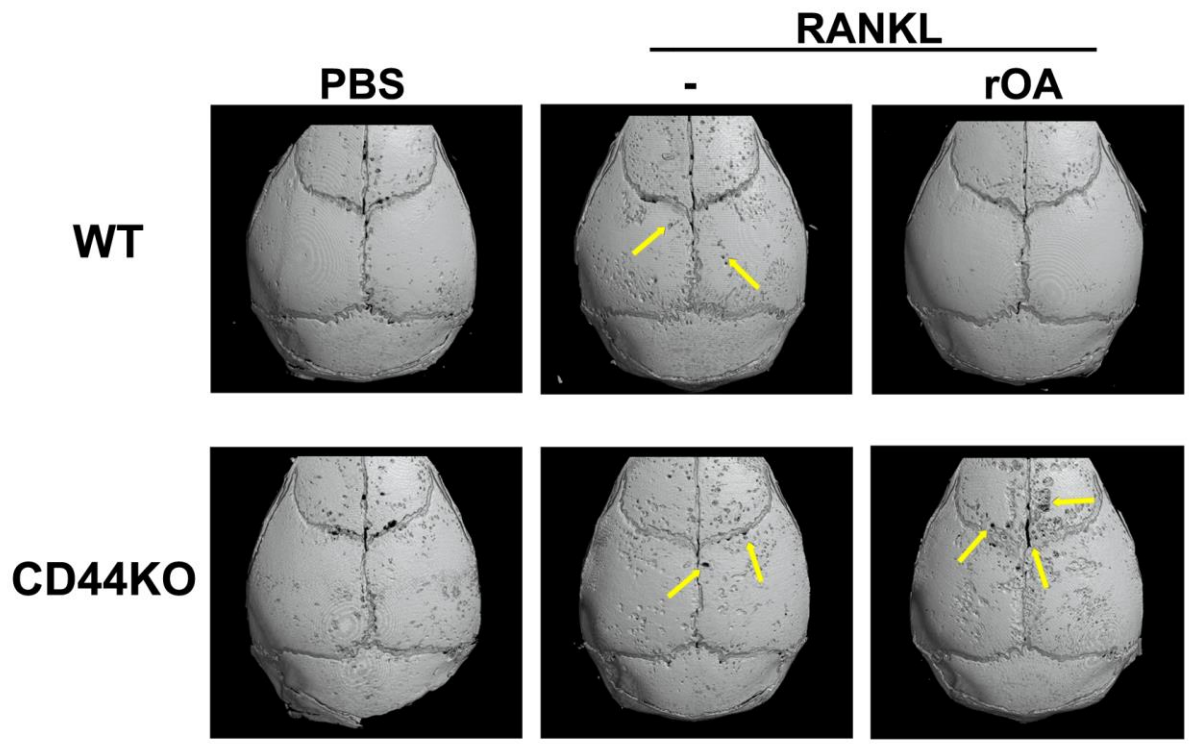


Figure 4.56: Recombinant Osteoactivin inhibits the resorption of osteoclasts through inhibition of osteoclast differentiation and recruitment *in vivo*. A collagen sponge coated with 30 µL of either PBS, RANKL (5 µg) alone, or RANKL and rOA (100 µg/mL) was placed on WT or CD44KO calvaria (n=5) for seven days. Calvaria were collected and scanned by microCT. Notice the increase in the number of pores (yellow arrows) in RANKL alone treated WT calvaria which are inhibited upon rOA treatment. In the CD44KO calvaria notice that upon rOA treatment, the number of pores do not seem to decrease.

4.22 *Recombinant Osteoactivin Stimulation of Osteoclast Resorption in vitro is Independent of CD44*

Osteoactivin has been shown to stimulate osteoclast resorption both *in vitro* and *in vivo* [7-9]. We wanted to determine if CD44 is involved in the osteoactivin-mediated stimulation of osteoclast resorption. The addition of Osteoactivin to WT osteoclasts significantly enhanced osteoclast resorption when cultured on Corning® discs (Figure 4.57). Interestingly, when Osteoactivin was added to CD44 deficient osteoclasts, a similar response as the wild type was observed. These results indicate that CD44 has no involvement in the Osteoactivin-mediated stimulation of osteoclast resorption on Corning® discs. To further elaborate, we cultured WT and CD44 deficient osteoclasts on cortical bovine slices and left them untreated (control) or treated with recombinant Osteoactivin. Wild type cells treated with Osteoactivin had smaller actin rings by immunoflourescent staining compared to untreated; however, there seemed to be no difference in actin ring size in CD44 deficient osteoclasts when treated with Osteoactivin (Figure 4.58). This indicates that Osteoactivin may be involved in regulating osteoclast sized potentially through CD44 mediated.

In order to determine the role of Osteoactivin in osteoclast resorption in WT and CD44 deficient osteoclasts, we treated osteoclasts with or without Osteoactivin for 48 hours and removed the osteoclasts. After the removal of the osteoclasts, we stained for lectin-HRP to observe eroded areas on the cortical slices. Lectin-HRP staining revealed WT osteoclasts treated with Osteoactivin had significantly high number of pits and increased total resorbed area (Figure 4.59). Interestingly, recombinant Osteoactivin treatment in CD44 deficient cells showed a similar pattern as WT, as Osteoactivin

increased pit number and total resorbed area. This indicates that Osteoactivin stimulates osteoclast resorption independent of CD44.

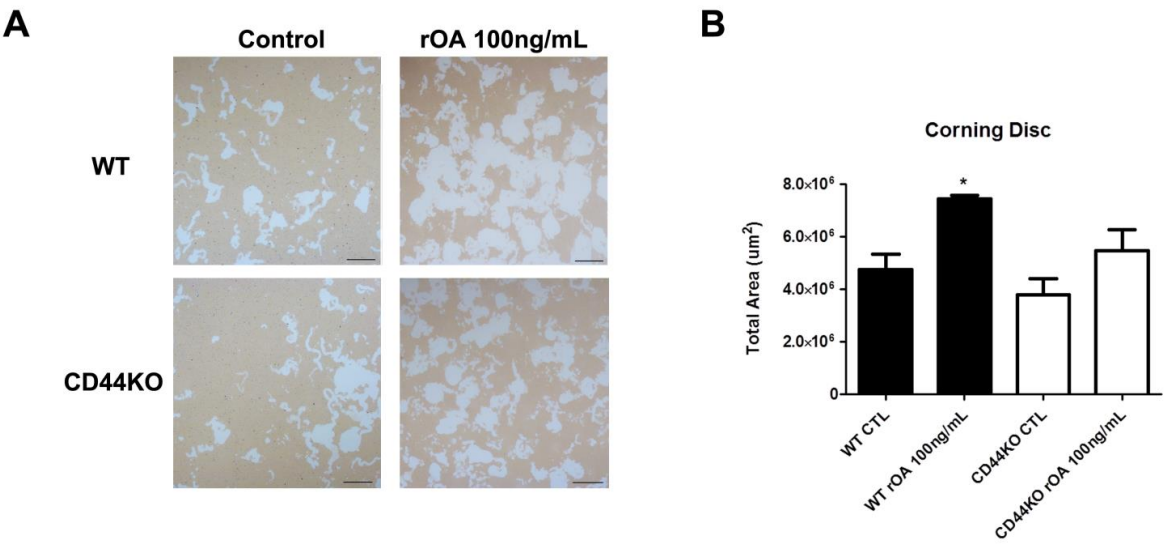


Figure 4.57: Recombinant Osteoactivin stimulates osteoclast resorption *in vitro* independent of CD44. BMM from WT and CD44KO cells were differentiated with M-CSF (20ng/mL) and RANKL (40ng/mL) alone (Control) or treated with recombinant osteoactivin (100 ng/mL) for 7 days to generate mature osteoclasts. (**A**) Microscopic images of Corning Discs revealing the resorption pits from the osteoclasts. (**B**) Analysis of the total area resorbed in WT and CD44KO cells untreated and treated with recombinant osteoactivin. Notice the increase in osteoclast resorption in WT rOA treated osteoclasts compared to control. The same trend is seen in the CD44KO cells indicating that CD44 does not play a role in osteoactivin mediated osteoclast resorption. The experiment was run in triplicates and repeated 3 times with similar results. Data presented in all graphs represent Mean ± SEM comparing untreated controls and rOA treated samples in WT and CD44KO. *****=$p<0.05$. Scale bars: 250 µm (Panel A).

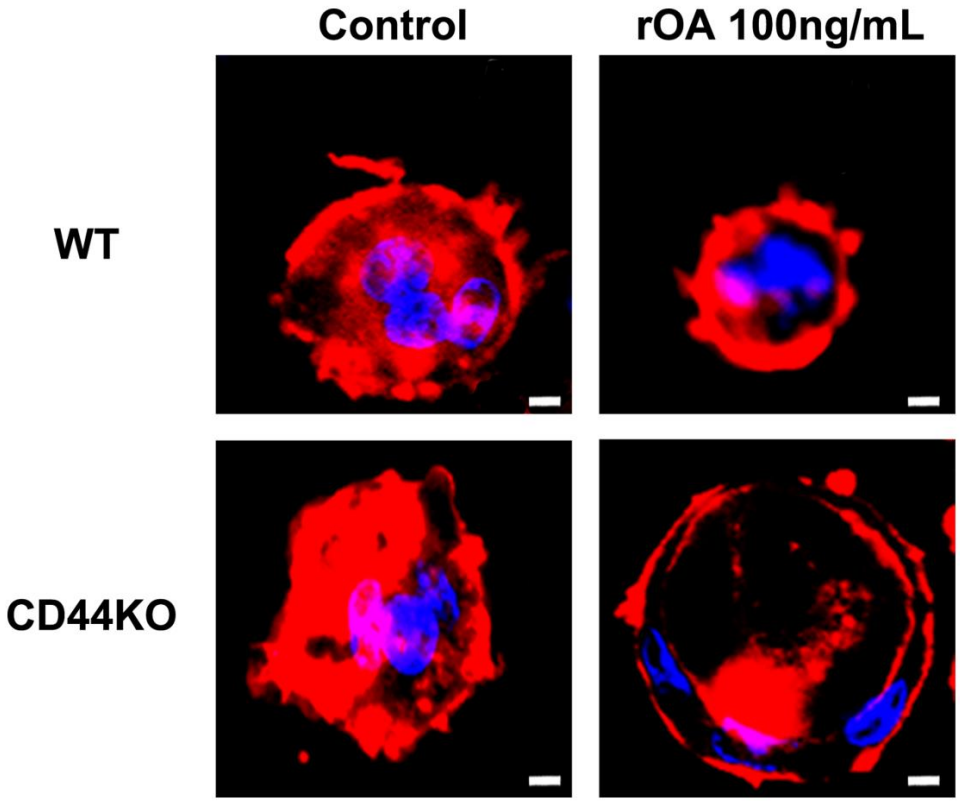


Figure 4.58: Osteoactivin treatment inhibits actin ring and osteoclast size on cells coated on cortical bovine slices. BMM from WT and CD44KO cells were differentiated with M-CSF and RANKL on collagen coated matrices for 5 days. After the 5th day, pre-osteoclasts were transferred onto bovine cortical slices for 48 hours along with a second dose of M-CSF and RANKL alone (Control) or in combination with recombinant osteoactivin. Notice that upon rOA treatment in WT cells the osteoclast actin ring and size are smaller compared to untreated control. This effect seems to be abrogated in CD44KO cells as there seems to be no change in osteoclast size or actin ring formation. Scale bars: 10 µm.

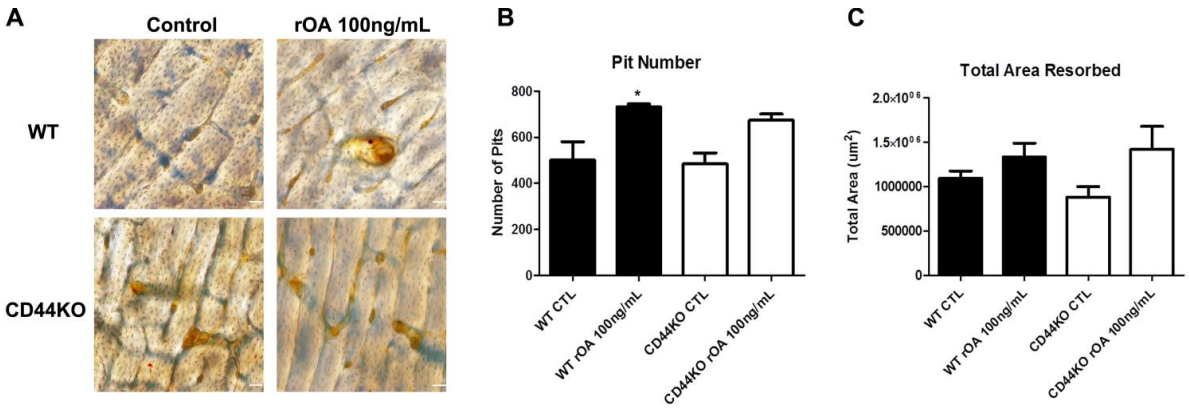


Figure 4.59: Recombinant Osteoactivin stimulates osteoclast resorption on cortical slices independent of CD44. BMM from WT and CD44KO cells were differentiated with M-CSF (20ng/mL) and RANKL (100ng/mL) on collagen coated matrices for 5 days. After the 5th day, pre-osteoclasts were transferred onto bovine cortical slices for 48 hours along with a second dose of M-CSF and RANKL alone (Control) or in combination with recombinant osteoactivin. Osteoclasts were removed and osteoclast pits were determined by Lectin HRP staining. (**A**) Microscopic images of Lectin HRP stained pits. (**B-C**) Analysis of Lectin HRP stained pits shows an increase in the number of pits (**B**) and total resorbed area (**C**) in rOA treated WT cells compared to control. A similar trend is seen in CD44KO osteoclasts. The experiment was run in triplicates. Data presented in all graphs represent Mean ± SEM. *****=$p<0.05$ comparing untreated controls and rOA treated samples in WT and CD44KO . Scale bars: 100 µm (Panel A).

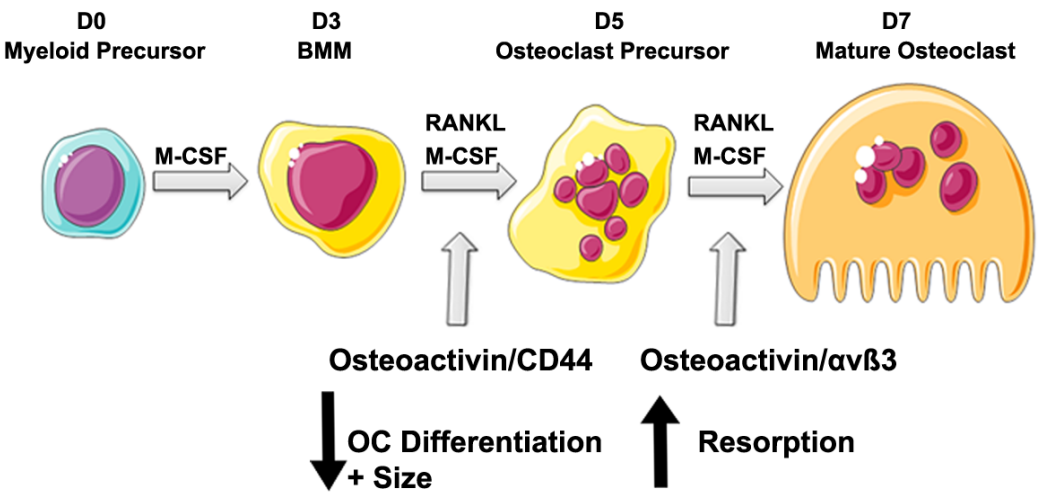


Figure 4.60: Schematic diagram indicating the role of Osteoactivin and its receptors during each specific stage of osteoclast differentiation. Our results indicate that Osteoactivin has dual roles during osteoclast differentiation. At an earlier stage, the Osteoactivin-mediated inhibition of osteoclast differentiation and size is CD44 dependent. At the mature stage, Osteoactivin-CD44 interaction had no effect on osteoclast resorption. The Osteoactivin-mediated increase in osteoclast resorption is most likely regulated through $\alpha_v\beta_3$ as previously described.

Chapter 5

Discussion

Summary

The main objective of this study was to determine the role of recombinant Osteoactivin in both osteoblasts and osteoclasts, and identify a novel signaling mechanism that related to its function in both cell types. We determined that Osteoactivin binds to CD44 in both osteoblasts and osteoclasts and regulates differentiation. For osteoblasts, Osteoactivin stimulated osteoblast differentiation and function in a CD44-ERK dependent mechanism. Interestingly Osteoactivin and CD44 were found to exist in the caveolin lipid raft and may also potentially regulate osteoblast differentiation through autophagy pathway. For osteoclasts, recombinant Osteoactivin inhibited osteoclast differentiation in a dose dependent manner. Similarly this Osteoactivin-mediated effect also seemed to be CD44 dependent; however, Osteoactivin-CD44 interaction had no effect on Osteoactivin-mediated upregulation of osteoclast resorption. Furthermore, in vivo results from a mouse osteolysis model revealed that Osteoactivin may inhibit osteoclasts differentiation in vivo. Overall these results support our hypothesis that Osteoactivin stimulates osteoblast differentiation while inhibiting osteoclast differentiation through a CD44 dependent mechanism.

5.1 Aim 1 The role of Osteoactivin and CD44 interaction during osteoblast differentiation

In this study, we determined the signaling pathway of Osteoactivin in osteoblasts. Previous literature has shown that Osteoactivin is important for osteoblast differentiation [4-6]. In this study, we showed that recombinant Osteoactivin was able to stimulate osteoblast matrix deposition and mineralization. Several other recombinant ligands and growth factors have shown to increase osteoblast differentiation in culture including BMP-2 [481], FGF-2 [482], Wnt3a [483], NOTCH [484], CCN-1 [485, 486], CCN-2 [487, 488] and BSP [489]. A previous report from our lab showed that overexpression of Osteoactivin increased osteoblast differentiation and function *in vitro and in vivo* [3, 5]. Our data supports this aspect that addition of Osteoactivin in culture enhances osteoblast differentiation and function.

Our data also indicates that CD44 may act as a novel receptor for Osteoactivin. CD44 has been shown to be expressed in both osteoblast and osteocytes and its expression increases during osteoblast differentiation [386, 394]. CD44 has been shown to have several ligands important for bone cell function which include hyaluronan, osteopontin, fibronectin, galectins, and serglycin.

Furthermore our data shows that CD44 deficient mice have significantly less bone both *in vivo* and *ex vivo*, indicating that CD44 may act as a positive regulator of osteoblast differentiation and function. Previous studies have shown that CD44 expression is very high in MSCs [490-492] and areas of fracture healing where expression was high in osteoprogenitor cells, osteoclasts, and osteocytes [407]. Furthermore, previous literature has found that knockdown of CD44 in dental pulp cells resulted in a reduction in ALP activity and mineralization *in vitro* [493]. This evidence supports our study that reduction or absence of CD44 results in an inhibition of osteoblast differentiation *in vitro*. Another study found that downregulation of CD44 in periodontal ligament cells resulted in reduced mineralized nodule formation and a reduction in osteoblast related gene expression of Intracellular adhesion molecule (ICAM), BMP-2, and FGF-1 [494]. This study supports our hypothesis that reduction of CD44 in osteoblasts results in a reduction of genes important for osteoblast differentiation and function. Furthermore, this study supports our hypothesis that upon addition of recombinant Osteoactivin to CD44 deficient osteoblasts, the Osteoactivin-mediated upregulation of Osterix and Col1α1 is abrogated. In addition, CD44 has been shown to downregulate Runx2 expression and activity in the ATDC5 chondrocyte cell line [495]. Taken together, our data clearly show that CD44 is a receptor of Osteoactivin function in osteoblasts. This is the first report to identify a novel receptor for Osteoactivin signaling in osteoblasts.

We showed that recombinant Osteoactivin treatment stimulated the phosphorylation of ERK and P38. The ERK pathway has been found to be critical for osteoblast differentiation and proper skeletal development as osteoblast-specific

dominant-negative (DN) transgenic ERK mice have significantly delayed skeletal development [496]. The authors speculate that ERK regulates Runx2 activity through an integrin-mediated mechanism indicating the importance of the ERK signaling pathway during osteoblast differentiation. P38 has also been shown to be an important regulator of osteoblast differentiation as inhibition of P38 through the use of inhibitors and DN transfection resulted in an impairment in ALP activity and mineralization [497].

Next, we sought to determine the signaling pathway downstream of Osteoactivin/CD44 interaction. We found that knockdown of CD44 and treatment with recombinant Osteoactivin in osteoblasts resulted in a decrease in ERK and P38 phosphorylation compared to scramble and recombinant Osteoactivin treated cells. CD44 has been shown to act as an important signal transduction molecule in several different cell types. A previous report has shown that ERK activation in cementifying fibroma cells of the jaw led to cell proliferation which was largely dependent on CD44 [498]. Furthermore, this report showed that Raf and MEK were activated upon stimulation of CD44 [498]. Raf and MEK have been shown to act upstream of ERK signaling. It will be interesting to determine if recombinant Osteoactivin stimulates Raf and MEK upstream of ERK signaling in osteoblasts. CD44 has also shown to be involved in P38 signaling. A previous study has shown that CD44 activation stimulates downstream P38 signaling resulting in the progression of cervical cancer [499]. Furthermore, hyaluronan was shown to inhibit IL-1β-induced MMP-13 expression through CD44-P38 dependent signaling in human arthritic chondrocytes [500]. This indicates that CD44 activation of P38 may serve as a protective function. It will be

interesting to determine if Osteoactivin-CD44 mediated activation of ERK or P38 has any protective function in bone.

Currently, there is little literature describing the signaling pathway of Osteoactivin mediated enhancement of osteoblast differentiation. Here, we show that CD44 binds to recombinant Osteoactivin in osteoblasts. CD44 has several different methods in which it can act as a signal transduction molecule. CD44 has shown to act as a co-receptor for other intrinsic kinase receptors including EGFR [501, 502], FGFR [503, 504], CXCR4 [505] and LRP6/Wnt signaling [506]. Each of these kinase receptors have been shown to play an important role in osteoblast differentiation and function. Interestingly, a previous study has shown that Osteoactivin may induce signaling through FGFR1 and ERK in MSCs [360]. It is possible that CD44 may act as a co-receptor and upon addition of Osteoactivin, CD44 may tether to FGFR1 and stimulate downstream ERK signaling. Future studies should focus on determining if Osteoactivin-CD44 binding stimulates downstream signaling directly, or requires another receptor.

Previous literature has shown that CD44 can also stimulate downstream signaling through its interaction with the cytoskeleton at its Ezrin-Radixin-Moesin (ERM) intracellular domain causing cytoskeletal rearrangement [507-509]. Actin cytoskeletal rearrangement has been shown to enhance osteoblast differentiation [510-512]. CD44 has also been shown to interact with integrin receptors within a large complex and stimulate downstream signaling [452, 513]. It may be possible that upon binding of Osteoactivin to CD44, CD44 may induce signaling through its interactions with the cytoskeleton and other integrin receptors. Furthermore a previous study has shown that Osteoactivin interacts with $\alpha_v\beta_1$ and stimulates osteoblast adhesion [367]. Furthermore,

another study revealed that Osteopontin, a ligand for CD44, stimulated MSC differentiation into osteoblasts and inhibitied adipocyte differentiation [514]. Therefore, it may be possible that CD44 may interact with $\alpha_v\beta_1$ and stimulate Osteoactivin-mediated downstream signaling and osteoblast differentiation (Figure 4.35). Future studies should focus on the role of Osteoactivin/CD44 interaction on actin cytoskeletal rearrangement.

Our data suggests that CD44 is important for Osteoactivin-mediated osteoblast differentiation *in vitro*. A previous report has shown that CD44 is necessary for Galectin-9-induced osteoblast differentiation through the Smad pathway [395]. This suggests that CD44 may be an important factor for stimulating osteoblast differentiation and function. Furthermore, another study revealed that CD44 stimulation along with $\alpha_v\beta_3$ stimulates Runx2 activation [515]. Previous literature has shown that Osteoactivin interacts with $\alpha_v\beta_3$ [8, 10]. In our study, recombinant Osteoactivin treatment to CD44 deficient osteoblasts resulted in an inhibition of osteoblast differentiation compared to WT treated cells. It will be interesting to determine if CD44 and $\alpha_v\beta_3$ interaction is the source behind Osteoactivin-mediated enhancement of osteoblast differentiation.

In our study, we have shown that upregulates Osterix, Col1α1, and Runx2 mRNA in WT osteoblasts. Interestingly, when recombinant Osteoactivin is added to CD44 deficient osteoblasts the upregulation of Osterix and Col1α1induced by Osteoactivin is unaffected; however, Runx2 remains upregulated. This indicates that the upregulation of Osterix and Col1α1 by Osteoactivin is CD44 dependent, but the upregulation of Runx2 by Osteoactivin is CD44 independent. Previous literature from our lab has shown that both overexpression of Osteoactivin or addition of recombinant Osteoactivin induce focal adhesion kinase (FAK) activation in C2C12 and MC3T3-E1 cells respectively [4,

367]. Furthermore, our lab has shown that overexpression of Osteoactivin in C2C12 cells stimulates osteoblast differentiation through the upregulation FAK activation of Runx2 and upon transfection of dominant-negative FAK (DN-FAK), this effect is abrogated [4]. Previous literature has shown that integrin activation stimulates downstream FAK-ERK-Runx2 signaling in osteoblasts [158]. Therefore, it may be possible that Osteoactivin stimulation of Runx2 may be mediated at least in part through integrin and FAK activation.

There is much attention being drawn to CD44 and Osteoactivin as repair factors. CD44 has been hypothesized to act as an important repair factor in a variety of tissues including skin [516], kidney [517-519], liver [520], and heart [521, 522]. CD44 has also been shown to play a large role in musculoskeletal repair. CD44 has been shown to play a large role in cartilage homeostasis [448, 523, 524]. Previous studies have shown that CD44 plays a role in chondrocyte matrix assembly [525, 526]. The role of CD44 in tissue injury repair may best be explained by the ability of CD44 to interact with certain growth factors and extracellular matrix proteins to stimulate MSC migration. A previous study has shown that CD44 and its interactions with hyaluronic acid and PDGF are necessary for stem cell migration [527]. Furthermore, MSCs have been shown to be recruited and migrate to areas of liver injury in a β_1 and CD44 dependent manner [528]. Preliminary data from our lab has shown that Osteoactivin stimulates MSC migration *in vitro* through a CD44 dependent mechanism (unpublished data). Furthermore, previous literature has shown that CD44 and Osteoactivin are both upregulated in fracture calluses [362, 407, 529]. Previous data from our lab and others have shown that Osteoactivin stimulates bone formation and angiogenesis *in vivo* in a mouse and rat

calvaria defect model [360, 363]. It will be interesting to determine if the interaction between CD44 and Osteoactivin can be translated *in vivo*. The use of fracture models in CD44 and Osteoactivin knockout models may help to identify the role of CD44 and Osteoactivin in bone repair.

5.2 Aim 2: The role of Osteoactivin in the osteoblast lipid raft

In this study, we sought to determine the relationship between Osteoactivin and the lipid raft in osteoblasts. Lipid rafts and caveolin proteins (Caveolin-1) have been shown to play an important role in osteoblasts. Caveolin-1 knockout mice were shown to have increased bone mass and stiffness [86]; however, another study revealed that knockdown or overexpression of Caveolin-1 inhibited or enhanced osteoblast mineralization, respectively [422]. Several other growth factors and extracellular matrix proteins important for osteoblast differentiation and function have been shown to be associated with lipid rafts. BMP receptors have been shown to co-localize within lipid rafts [530, 531]. Interestingly, BMP-2 treatment in osteoblasts has shown to enhance the incorporation of β_1 integrin into lipid rafts resulting in the promotion of cell migration [430]. Furthermore, FGFR2 activation has been shown to act as a pro-survival factor in osteoblasts [429]. Another study has shown that interaction between the lipid raft and Galectin-9 stimulates osteoblast proliferation through a Src/ERK mediated mechanism [431]. It will be interesting to determine whether Osteoactivin function is mediated through lipid rafts in osteoblasts.

Our lab has shown that Osteoactivin binds to and interacts with CD44 in osteoblasts. CD44 has been shown to co-localize with other kinase receptors and

stimulate downstream signaling. A previous study has shown that transdifferentiation of fibroblast to myofibroblast is dependent upon Epidermal Growth Factor Receptor (EGFR) and CD44 co-localization into lipid rafts [450]. Furthermore, knockdown of CD44 and disruption of the lipid rafts by nystatin resulted in an inhibition in ERK and CaMKII signaling in fibroblasts [450]. Another study revealed that upon CD44 engagement with another ligand, CD44 and Caveolin-1 co-localize within the lipid raft and recruit β_1 integrin and Src to facilitate signaling resulting in increased cell survival [452]. It will be interesting to determine the role of CD44-Osteoactivin-lipid raft signaling complex in osteoblasts and how it relates to osteoblast differentiation and function.

Currently, there exists the idea that cross-talk among signaling complex exists to facilitate downstream signaling and function. Our data indicates that disruption of the lipid raft inhibits downstream ERK signaling in osteoblasts. Furthermore, CD44 engagement with Osteoactivin results in co-localization into lipid rafts. Previous data from our lab has shown that Osteoactivin interacts with β_1 integrin and facilitates downstream ERK and FAK signaling [367]. Therefore, it may be plausible to assume that Osteoactivin along with CD44 form a complex within the lipid raft that stimulates downstream signaling. Since Osteoactivin has been shown to stimulate multiple signaling pathways, it may also be possible that cross-talk exists among signaling complexes. A previous study has shown that cross-talk between Caveolin-1/ERK and Wnt/β-catenin is important for osteocyte viability under mechanical stress [532]. Further investigation is needed to determine if cross-talk among signaling pathways exists upon Osteoactivin stimulation.

Our results indicate that recombinant Osteoactivin interacts with both CD44 and Caveolin-1 in the lipid raft complex. Previous literature has shown that lipid rafts can be endocytosed into the cell resulting in the stimulation of cell signaling [533-536]. Furthermore, CD44 has been extensively shown to be involved in caveolin-mediated endocytosis of hyaluronan resulting in intracellular signaling [537-540]. Currently, there is an accumulation of evidence that indicates that endocytosis may play a role in osteoblast signaling and differentiation. A previous study has shown that inhibition of endocytic processes during BMP-2 stimulation resulted in impaired Smad 1/5/8 signaling and inhibited osteoblast differentiation [541]. Furthermore, endocytosis has been shown to be important for extracellular matrix remodeling, degradation, and receptor recycling in osteoblasts [542-544]. During endocytosis, the actin cytoskeleton undergoes rearrangement in order to bring the complex into the cell [545]. Since CD44 has been shown to associate with and transduce signaling through the lipid raft and actin cytoskeleton [451, 546, 547] it may be plausible that Osteoactivin,CD44, and the lipid raft complex may undergo endocytosis and internalization into the osteoblast resulting in the stimulation of cell signaling. It will be interesting to determine if Osteoactivin undergoes endocytosis upon CD44 binding and whether disruption of this process regulates Osteoactivin mediated osteoblast differentiation.

One of the downstream processes of the caveolin-lipid raft complex (caveosome) after internalization, is to undergo endosome formation. Once the complex undergoes endosome formation, the complex is either recycled to the cell membrane, or undergoes degradation in the autophagosome [459, 548, 549]. The autophagosome will eventually fuse with an intracellular lysosome resulting in an autophagolysosome in a process

known as autophagy [550]. Our results show that Osteoactivin may undergo autophagy in osteoblasts through the conversion of LC3B-I to LC3B-II. Autophagy has become an emerging field in musculoskeletal biology. Previous studies have shown that suppression of autophagy results in osteopenia, decreased bone mineral density, and inhibition of terminal osteoblast differentiation, [463, 464]. Furthermore, a previous study has shown that bioactive silica nanoparticles promote osteoblast differentiation through stimulation of autophagy [461]. Interestingly the authors of this study showed that these nanoparticles enter the cell through a caveolae-mediated endocytosis process through ERK stimulation. This may explain what might be occurring in the Osteoactivin mediated process involving CD44, the lipid raft, and endocytosis. Future studies should focus on the role of Osteoactvin and autophagy during osteoblast differentiation and function.

The role of caveolins in the disease process, in particular musculoskeletal diseases, is only recently becoming understood. Caveolin-1 is differentially expressed in certain tissues during injury repair. For example, Caveolin-1 has a positive role in cutaneous skin wound healing as Caveolin-1 knockout mice have slower wound healing compared to controls [551, 552]. During muscle regeneration, Caveolin-1 has been shown to be downregulated during injury repair both *in vitro* and *in vivo* [435, 553]. In chondrocytes, lipid rafts have been shown to be important for chondrogenesis through neogenin and BMP receptor interaction [554]. Interestingly, patients with bone fracture have increase Caveolin-1 levels in peripheral blood mononuclear cells (PBMCs) [436]. Furthermore, overexpression of Caveolin-1 in human MSCs resulted in an inhibition of adipogenic differentiation. This data indicates that Caveolin-1 may be an important

regulator of skeletal injury. Future directions should focus on the role of Osteoactivin and Caveolin-1 during fracture repair.

5.3 Aim3: The role of Osteoactivin-CD44 interaction during osteoclastogenesis

In this study, we showed that recombinant Osteoactivin inhibits osteoclast differentiation and size by downregulating osteoclast specific genes. Our lab was the first to show that mutation in Osteoactivin results in increased osteoclast differentiation but inhibits resorption [7]. Controversially, other groups have published that Osteoactivin stimulates both osteoclast differentiation and function [8, 9]. This may be explained by the fact that Osteoactivin may regulate osteoclasts in a stage specific manner. Previous results generated from our lab have shown that addition of recombinant Osteoactivin has different effects on osteoclast differentiation and function (unpublished results). This may explain the discrepancy observed between our group and others.

In this study, we demonstrated that recombinant Osteoactivin inhibits osteoclast differentiation *in vitro* and *in vivo* through a CD44-ERK dependent mechanism. Previous data from our lab has shown that mutation in Osteoactivin results in enhanced osteoclast differentiation and inhibits bone resorption [7]. Furthermore, mutation in Osteoactivin resulted in an increase in the MAPK pathway. The MAPK pathway has extensively been shown to be important for osteoclast differentiation [555-557]. In this study, RANKL was used to stimulate members of the MAPK, NF_KB, and AKT pathway in osteoclasts. Our data indicates that recombinant Osteoactivin inhibits AKT and ERK activation during RANKL mediated osteoclast signaling. Previous literature has shown that TNF-α and IL-1α stimulate osteoclast survival through AKT and ERK signaling [558,

559]. Furthermore, PI3K was shown to coordinate the activity of both MEK/ERK and AKT to maintain osteoclast survival [560]. It will be interesting to determine if recombinant Osteoactivin plays any role in either MEK or PI3K signaling and if recombinant Osteoactivin regulates osteoclast survival through the MEK/ERK or AKT pathways in osteoclasts.

In the current study, we have shown that Osteoactivin interacts with CD44 in osteoclasts. Previous studies have shown that absence of CD44 has no effect on osteoclastogenesis [393]; however, when challenged with an inflammatory cytokine such as TNF-α, develop inflammatory bone loss [391]. Furthermore, engagement of CD44 with hyaluronan was found to inhibit MMP-9 mRNA expression and result in the inhibition of osteoclast motility [405]. Similarly, our results show that Osteoactivin binds to CD44 in osteoclasts and that Osteoactivin mediated inhibition of osteoclasts is CD44 dependent. CD44 has also been shown to be an important fusion marker [561], and upon engagement with another ligand, inhibits multinucleation [406]. Similarly, our data shows that recombinant Osteoactivin inhibits osteoclast size which seems to be CD44 dependent. CD44 has been shown to play a role in osteoclast migration and adhesion [562]. We did not study the role of Osteoactivin on osteoclast motility. It will be interesting to determine if Osteoactivin plays a role in osteoclast motility.

CD44 has shown to play a role in osteoclast resorption. Several sources of literature have shown that CD44 along with $\alpha_v\beta_3$ integrin regulate osteoclast motility and bone resorption [398, 400, 563]. Osteoactivin has shown to bind to $\alpha_v\beta_3$ and is believed to be responsible for osteoclast mediated resorption [8, 10]. In our study, we reveal that Osteoactivin binds to CD44; however, our data suggests that CD44 does not play a role

in osteoclast mediated resorption *in vitro*. This may be explained by the fact that previous literature has shown that CD44 and $\alpha_v\beta_3$ integrin organize two functionally distinct domains in osteoclasts [564]. In this study, the authors reveal that CD44 occupies the osteoclast podosome core, structures important for differentiation, adhesion and migration, while $\alpha_v\beta_3$ integrin occupies the outer F-actin cloud, important for matrix degradation. Therefore it may be possible that Osteoactivin binds to CD44 and inhibits osteoclast differentiation and fusion, but also binds to $\alpha_v\beta_3$ integrin and stimulates osteoclast resorption *in vitro* (Figure 4.60). Future studies should focus on particular interactions and binding specificities between Osteoactivin, CD44, and $\alpha_v\beta_3$ in modulating osteoclast function.

Our data suggests that recombinant Osteoactivin inhibits osteoclast differentiation, but stimulates resorption *in vitro*. Several other extracellular matrix proteins have shown to have this similar effect on osteoclasts. Fibrillin-1 has been shown to have dual roles in regulating osteoclast differentiation and function [565]. In this study, recombinant N-terminal Fibrillin-1 was shown to inhibit osteoclast formation but stimulate its function *in vitro*. Previous literature has also shown that fibronectin inhibits osteoclast differentiation activity, but stimulates function through IL-1β and nitric oxide [69]. Interestingly, fibronectin has been found to bind CD44 through its heparin binding domain [566]. It will be interesting to determine if CD44, Osteoactivin, and fibronectin have any interaction or involvement in osteoclast differentiation and function.

Our data suggests that recombinant Osteoactivin inhibits osteoclast differentiation and recruitment in an inflammatory *in vivo* osteolysis model. Previous literature has shown several different anti-inflammatory components that suppress

osteoclast formation in vivo [477-479]. It is interesting to note that recombinant Osteoactivin has a negative response on osteoclast resorption *in vivo*, yet stimulated its function *in vitro*. There may be two separate explanations seen in this phenomenon. First, Osteoactivin may be inhibiting the recruitment and differentiation of osteoclasts directly resulting in a reduced number of osteoclasts and less resorption. Secondly, the results seen *in vivo* may be indirectly due to the effect of Osteoactivin on osteoblasts. Previous data from our lab has shown that addition of recombinant Osteoactivin in osteoblasts results in the reduction of RANKL and other inflammatory cytokines (unpublished data). This is also supported by the fact that mutation in Osteoactivin results in an increase in RANKL expression [7]. Therefore, it may be possible that Osteoactivin may be indirectly affecting the osteoclasts by reducing the production of RANKL and other inflammatory cytokines secreted by the osteoblasts. Future studies should focus on the dual roles of Osteoactivin in osteoblasts and osteoclasts using tissue specific transgenic or knockout animals to define the role of Osteoactivin in these cell types.

Interestingly, deficiency of CD44 resulted in the abrogation of Osteoactivin mediated inhibition of osteoclast differentiation and recruitment *in vivo*. Previous studies have shown that CD44 has a potential role as an anti-inflammatory component in different tissue types [567-569]. A previous study has shown that CD44 can act as a critical inhibitor for joint destruction and inflammatory bone loss [391]. Furthermore, the anti-inflammatory response through CD44 has been linked to TGF-β [570-572]. Interestingly, Osteoactivin has been shown to regulate TGF-β levels as transgenic mice overexpressing Osteoactivin have increased levels of TGF-β [5]. It will be interesting to

determine if there is a link between Osteoactivin, CD44, and TGF-β in reducing the inflammatory response *in vivo*.

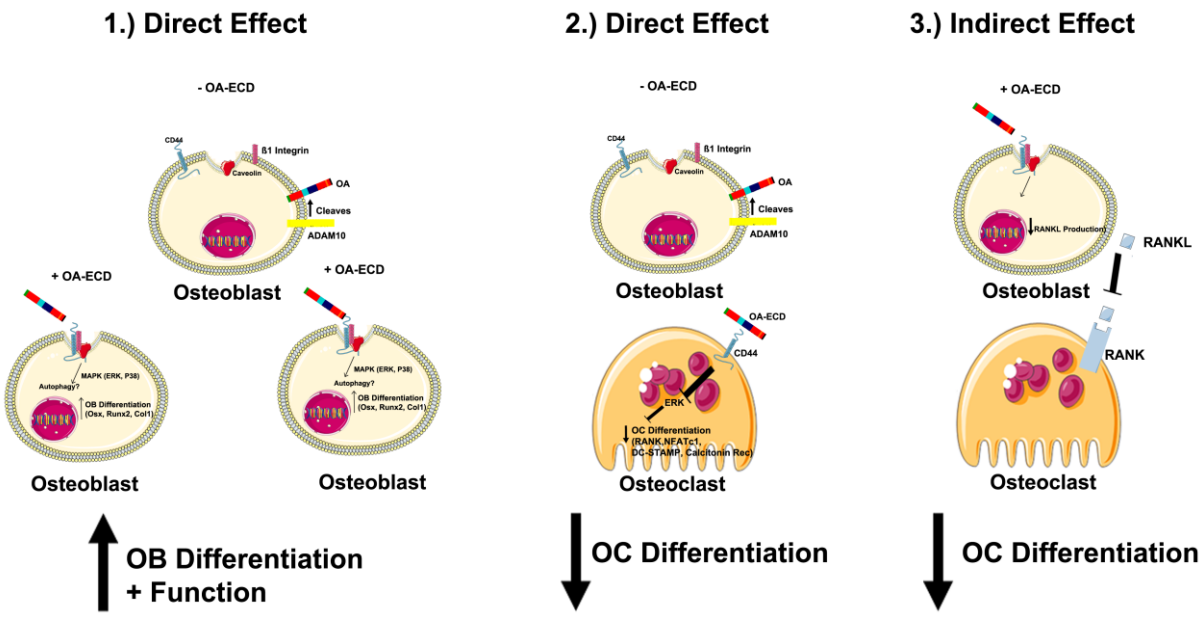


Figure 5.1: Schematic diagram depicting our overall hypothesis of the role of Osteoactivin-mediated regulation of both osteoblast and osteoclast. (1.) Osteoactivin has a direct effect on osteoblast differentiation and function. Osteoactivin is shed from the membrane and effects other MSCs and osteoblasts within the bone microenvironment. **(2.)** Osteoactivin has a direct effect on osteoclast differentiation. Osteoactivin shed from the osteoblasts binds to CD44 in the osteoclasts and inhibits osteoclast differentiation and size. **(3.)** Osteoactivin has an indirect effect on osteoclast differentiation through osteoblast regulation. Preliminary results indicate that Osteoactivin downregulates RANKL expression in osteoblasts. This results in a lower concentration of available RANKL in the matrix resulting in an inhibition of osteoclast differentiation. All of these mechanisms support our overall hypothesis that Osteoactivin increases bone formation by increasing osteoblast and inhibiting osteoclast differentiation.

Overall Summary and Conclusions

In this study, we have shown that recombinant Osteoactivin stimulates MSC and osteoblast differentiation *in vitro*. This was shown by the increase observed in ALP staining and activity as well as mineralization. Furthermore, recombinant Osteoactivin was shown to upregulate several osteoblast specific genes by qPCR and luciferase analyses. We identified CD44 as a potential receptor responsible for the Osteoactivin-mediated increase in osteoblast differentiation and function.

In order to understand the role of Osteoactivin-CD44 in osteoblast differentiation, we wanted to determine the role of CD44 in skeletal homeostasis. CD44 deficient mice had significantly less bone *in vivo* shown by microCT, histomorphometry, and serum analysis. *Ex-vivo* analysis revealed a reduction in osteoblast-related genes in the CD44 deficient mice compared to controls. We concluded that the bone phenotype observed in the CD44 deficient mice was osteoblast, not osteoclast mediated.

Next we sought to determine the role of Osteoactivin and CD44 in osteoblast cell signaling. We demonstrated that addition of recombinant Osteoactivin resulted in an increase in pERK and pP38 levels in a dose and time dependent manner. This increase was abrogated when cells were transfected with CD44 siRNA. To demonstrate the role of Osteoactivin and CD44 during osteoblast differentiation, we treated recombinant Osteoactivin to WT and CD44 deficient MSCs. Recombinant Osteoactivin enhanced ALP staining and activity in the WT MSCs, however, this effect was abrogated in CD44 deficient MSCs. Similarly, recombinant Osteoactivin addition upregulated osteoblast

related genes Col1α1 and Osterix; however, this was also abrogated in CD44 deficient cells.

Next, we sought to determine if Osteoactivin has any role in the lipid raft in osteoblasts. Our data suggests that Osteoactivin interacts with the lipid raft. Furthermore, recombinant Osteoactivin treatment results in the accumulation of CD44 into lipid rafts. Lastly, disruption of lipid rafts results in the inhibition of ERK phosphorylation.

In conclusion, we sought to determine the role of recombinant Osteoactivin on osteoclast differentiation and signaling. Our data suggests the Osteoactivin inhibits osteoclast differentiation shown by TRAP staining and activity and gene expression. Osteoactivin treatment resulted in the inhibition of RANKL-mediated ERK signaling which seems in part to be mediated in part through CD44. Furthermore, recombinant Osteoactivin inhibited RANKL-induced osteoclast differentiation and recruitment *in vivo* using an osteolysis model. Interestingly, this effect was abrogated in CD44 deficient animals indicating that Osteoactivin and CD44 may play a role in inhibiting inflammatory-induced bone loss.

Overall, this project has shown that Osteoactivin acts as a positive regulator for bone formation. This concept may introduce the idea for the use of Osteoactivin as a novel therapeutic to help treat bone disease. Potential therapies may include systemic and local delivery of Osteoactivin, spinal fusion, and/or combined therapy with Osteoactivin and stem cells/bone graft. Future research should focus on specific applications of treatment for Osteoactivin in treating specific skeletal pathologies.

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